# A POTENTIAL PHYTOMEDICINE FOR OBESITY FROM THE LEAVES OF *Dalbergia sissoo* Roxb. USING ZEBRAFISH (*Danio rerio*) - A METABOLOMIC APPROACH FOR FUTURE HERBAL DRUG DEVELOPMENT

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## CERTIFICATE

This is to certify that the dissertation entitled **"A POTENTIAL PHYTOMEDICINE FOR OBESITY FROM THE LEAVES OF *Dalbergia sissoo* Roxb. USING ZEBRAFISH (*Danio rerio*) - A METABOLOMIC APPROACH FOR FUTURE HERBAL DRUG DEVELOPMENT"** was carried out by **Ms. G. Josephin Nerling Rashida**, in the Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai – 625 020, in partial fulfillment of the requirement for the **Degree of Master of Pharmacy** (Pharmacognosy). This dissertation is forwarded to the Controller of Examination, The Tamilnadu Dr. M.G.R. Medical University, Chennai.

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## Abbreviations

% - Percentage

$DDH_2O$ -Double distilled water

g -Gram

Hcl -Hydrochloric acid

$HgCl_2$ -Mercuric chloride

L -Litre

M -Molar

mg/L -Miligram per litre

min -Minute

mL –Millilitre

µg – Microgram

T.S- Transverse section

MS -Murashige and Skoog (1962) medium

BM-Basal Media

NAA -Napthalene acetic acid

NaOCl -Sodium hypochlorite

NaOH -Sodium hydroxide

Rpm -Rotation per minutes

PGR -Plant growth regulator

v/v -Percent “volume in volume”

w/v -Percent “weigh in volume”

UV -Ultra Violet

NMR-Nuclear Magnetic Resonance

EI MS – Electron Impact mass spectroscophy

DPPH-1,1-diphenyl-2-picrylhydrazyl

DSEE-*Dalbergia sissoo* ethanolic extract of leaves

DSIF- *Dalbergia sissoo* isolated fraction

DSEEC-*Dalbergia sissoo* ethanolic extract of callus

Dpf-Days post fertilization

Hpf – Hours post fertilization

HC- High cholesterol

HFD – High fat diet

ND – Normal diet

BID – Bis in die

TC- Total cholesterol TC

TG-Triglyceride

GOT-Glutamate oxaloacetate transaminase

GPT -Glutamate pyruvate transaminase

BMI - Body mass index

# CHAPTER I

# INTRODUCTION

**Obesity** is a medical condition in which excess body fat has accumulated to the extent that it may have an adverse effect on health, leading to reduced life expectancy and/or increased health problems [1, 2].

Body mass index (BMI), a measurement which compares weight and height, defines people as overweight (pre-obese) if their BMI is between 25 and 30 $kg/m^2$, and obese when it is greater than 30 $kg/m^2$.Obesity is a leading preventable cause of death worldwide, with increasing prevalence in adults and children, and medical specialist view it as one of the most serious public health problems of the $21^{st}$ century [3].

BMI is calculated by dividing the subject's mass by the square of his or her height, typically expressed either in metric units:

**Metric: *BMI = kilograms / meters*$^2$**

## ADIPOSE TISSUE AND OBESITY

Adipocyte (fat cell) is the major component of adipose tissue that is known as loose connective tissue or fat tissue that function as an energy storage site in the form of triglyceride [10]. Adipose tissue plays an important role in maintaining the free fatty acid levels and triglycerides in circulation. It has been demonstrated that an increased amount of adipose tissue is related to obesity by **hyperplasia or hypertrophy** of the adipocyte. Hyperplasia, which is an increase in the number of adipocytes, this occurs by pre-adipocyte differentiating into adipocyte [4].

Adipose tissue mass can also increase by hypertrophic growth, which is an increase in the size of adipocyte [11]. Although obesity is associated to increase of body weight, the definition of obesity is not dependent on body weight but on the amount of body fat, specifically adipose tissue. In other words, obesity is a condition of abnormal large amount of fat stored in adipose tissue and an increase in bodyweight is generally associated with an increased risk of excessive fat-related metabolic diseases (EFRMD) and chronic diseases, including Type 2 diabetes mellitus, hypertension and dyslipidemia [12, 13,14].

There are two types of adipose tissue,

- Brown adipose tissue
- White adipose tissue

**White adipose tissue** can compose up to 25% of body weight in men and women and its main purpose is the storage site for fat in the form of triglycerides and cholesterol ester. Brown adipose tissue is found mainly in newborn or hibernating mammals because its primary purpose is to generate body heat [6, 7].

As for white adipocytes, it serves for three functions such as heat insulation, mechanical cushion and source of energy. White adipose tissues can be found mostly in perivascular, inter muscular, peritoneal, retroperitoneal, and subcutaneous. It also secretes resistin, adiponectin and leptin. In male mouse, adjacent to the epididymis and testes, there deposited large amount of intra-abdominal white adipocytes. Adipocytes stores along the uterine horns in female mouse are known as the parametrial fat pads. When mice grow into adulthood, its brown fat is best easily observed. **Brown adipocytes** are found in dorsal of the thorax, aorta of the heart and also in the hilus of the kidney [15].

Research has shown that obese people who have more abdominal fat are more prone to get cardiovascular disease, diabetes and metabolic syndrome [16, 17]. Although the physiological role of brown adipose tissue in humans is debated, it is reported that brown adipose tissue in rodents has an important role in the prevention and therapy of obesity [18].

In conclusion, it is possible to inhibit adipose tissue mass by decreasing the adipose tissue mass as well as adipocyte number [19]. In addition, to decrease body weight and lower the risk of several chronic diseases- especially metabolic syndrome can also be achieved by lowering the abdominal fat.

**OBESITY AND HUMAN HEALTH**

Obesity results from energy imbalance between energy intake and energy expenditure over a period of time. Increased energy intake (calories) with the decline of physical activity promotes weight gain, body fat storage and adiposity growth in a pathologic direction [4].

In addition, obesity has been predicted to be the number one health problem globally by the year 2025 and thought to be overtaking cigarette smoking soon to become the leading cause of death in the USA [8, 9].

Several chronic diseases are demonstrated related to obesity which including the following [5, 6,7 ].

**CAUSES OF OBESITY**

Obesity caused by many factors which may affect the risk of coming into an imbalanced state, such as genetic/epigenetic vulnerability and many other ones, some of which are discussed below.

***Eating habits****:* There is much discussion about eating more "fast foods", larger portion sizes, foods with higher fat and sugar content, less fruits and vegetables, more sweets, soft drinks and snacks. The comfort life would be one of the major culprits for today's obesity epidemic [20, 21]. A prospective study in children aged 11–12 years found that this consumption was associated with a 60% increased risk of obesity [22, 23].

***Low physical activity****:* Television viewing and other sedentary behaviours increase the risk of obesity. The technological options for enjoyable sedentary behaviours are increasing. Watching television has been directly linked to obesity, with a rate of obesity that is 8.3 times greater among who watched more than 5 hour of television per day compared with those who watch up to 2 hour per day [24]. Researchers suggest that obese are slightly less physically active; however, energy expenditure due to physical activity does not seem to differ [25-28].

***Heredity***: i.e. parental obesity has been identified as a major risk factor for obesity, probably due to a combination of genetic, epigenetic, social and environmental factors [29, 30]. Social factors seem to be of some importance for BMI heritability since associations have been found between the BMI of adoptees and adoptive parents [31]. Children with two obese parents have a higher risk of obesity than those with one or no obese parent [32].

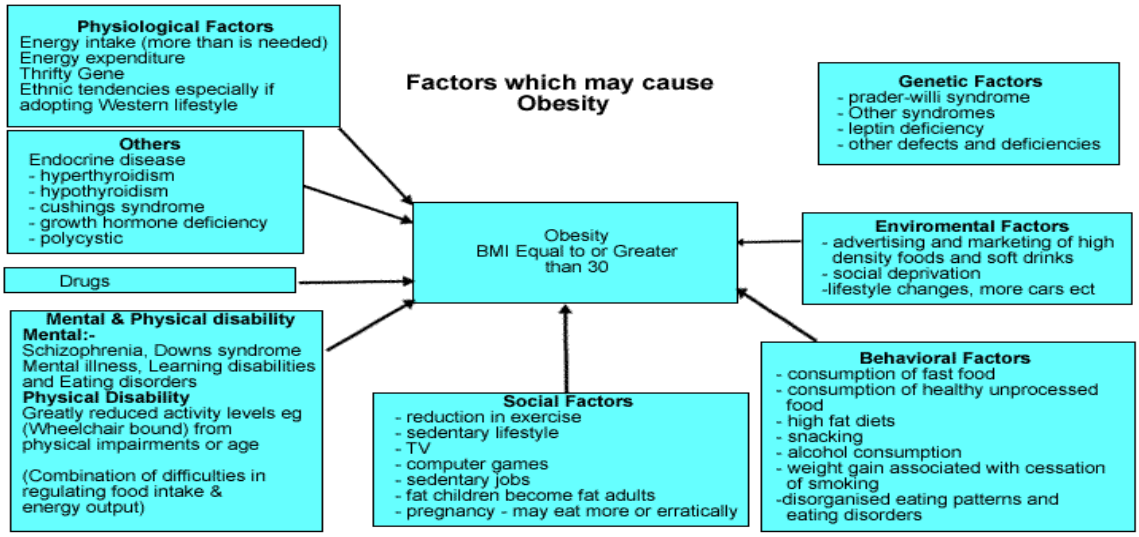
***Genetic factors:*** have been suggested to affect behavioral factors by altering appetite or physical activity patterns. The first of common mutations found were in the melanocortin-4-receptor (MC4R), affecting less than 5% of obese children [33]. The most important one found so far is the fat mass and obesity-associated gene, FTO. For a single set the risk is 38% [34, 35, 36].

***Sleep:*** Some studies have shown that fewer hours of sleep are associated with an increased BMI both in children and adults [37, 38]. Children 5–10 years old with the least amount of sleep, 8–10 hours per night, had a 3.45–4.9 times higher risk of being classified overweight than children sleeping longer [39-41] studied indicate that sleep duration and regularity affect body weight [42]. Decreased leptin and increased ghrelin levels are associated with sleep deprivation and both hormone changes may induce increased food intake [39, 40].

***Viruses***: In several animal models researchers have found that viruses have been shown to cause obesity In US adults. Atkins *et al.* found that 30% were infected with **human adenovirus-36 (Ad-36)** and had 9 units higher BMI compared with those not infected [43]. The same pattern was seen in obese Korean children with 30% positive and significantly higher BMI and waist circumferences [44]. Thus, Ad-36 may have a function in the obesity epidemic.

***Epigenetics****:* Environmental factors may affect DNA activity without changing the DNA molecule itself. Small molecules can bind to the DNA strain and thereby reduce the activity of specific genes. These genetic modifications may be hereditary. Thus, environmental factors *in utero* can have long-term effects and even affect the next generation [45].

"*Epigenetics has always been all the weird and wonderful things that can't be explained by genetics."* Denise Barlow (Vienna, Austria)




**Drug molecules for obesity:**

Pharmacological agents are potential adjuncts to behavioral interventions for severely obese [48] but, unfortunately, no pharmacological treatments are available today for children and adolescents.

Drugs with a direct effect on weight reduction can be divided into:

- Drugs acting in the central nervous system, interfering with neurons involved in appetite and satiety regulation.
- Drugs locally acting in the intestine by inhibiting uptake of nutrients.
- Drugs acting in the central nervous system or peripheral-acting drugs aimed at increasing energy expenditure.

***Orlistat***

Orlistat acts locally in the gut lumen by inhibiting gastrointestinal lipase. This enzyme normally breaks down triglycerides in the intestine to make them absorbable. Thereby, one reduces the uptake of consumed fat in the diet by 30%. The unabsorbed fat passes through the bowel, resulting in fatty stools. Therefore, the primary side effects if one

eats too much fat are steatorrhoea, i.e. oily, loose stools. Orlistat may also interfere with the absorption of fat-soluble vitamins (A, D, E and K). It is therefore recommended that a daily multivitamin supplement should be taken during treatment [49].

***Sibutramine***

Sibutramine works in the central nervous system by reducing serotonin and noradrenaline reuptake. Sibutramine thereby reduces the appetite, to some extent, increases energy expenditure. The most common side effects are increased blood pressure and heart rate, dry mouth, insomnia, dizziness and constipation [50]. Since increased cardiovascular events and stroke have been observed during sibutramine treatment in adults it has been withdrawn from the market in major parts of the world.

***Rimonabant***

Rimonabant is a cannabinoid-1 receptor blocker and is thereby considered to be an appetite suppressant. It works by blocking a cellular receptor in the endo cannabinoid system of the brain, which is believed to influence the regulation of body weight, glucose and lipid metabolism. Approval of the drug was officially withdrawn in January 2009 due to the possibility of serious psychiatric problems and even suicide.

***Metformin***

Metformin is an old and proven anti-diabetic drug. It is the first-line drug for the treatment of type 2 diabetes and it is not marketed as a weight loss medication. More recently it has been observed that it has a positive effect on weight. Side-effects are few and consist mainly in gastrointestinal distress, especially at the beginning of treatment.

***Ephedrine/caffeine***

In some countries the combination of caffeine and ephedrine is approved for obesity treatment. There is limited support for this indication in adults [51, 52].

***Very low calorie diet***

A very low calorie diet (VLCD) is defined as a protein-sparing diet with only 600-800 kcal per day. VLCD also contains the recommended amounts of nutrients such as vitamins and minerals to be the sole energy and nutrition in the treatment of overweight and obesity. A low calorie diet (LCD) is a similar diet with 900–1200 kcal per day.

***Surgery***

Bariatric surgery (predominantly **Roux-en-Y gastric bypass**) for the management of severe adult obesity has been shown to be effective in maintaining significant weight loss and improvements in many of the medical complications [53]. There are many ongoing studies for adolescents, but it is still unclear if **bariatric surgery** is an option for obese adolescents. One randomized controlled study has been published in which the adjustable gastric banding was tested versus behavioral treatment [54]. Although 28% had complications requiring surgery, the two-year results are very promising. [55, 56, 57]

**BACKGROUND OF THE STUDY**

Obesity is one of the major public health problems in the United States and other developed countries. It is believed to be associated with several major chronic diseases such as cardiovascular diseases, diabetes, and cancers. One of the national health goals for the year 2010 is, to reduce the prevalence of obesity among adults to less than fifteen percentage [1].

Current research appears to continuously widen the horizon of possible factors of importance for the obesity epidemic seen today.

Although obesity is one of the major health problems in the United States, there is not an effective drug to treat obesity because they all have undesirable side effects. However, it is believed that botanicals provide a safer and natural way to human body in both pharmaceutical and nutraceutical aspects.

**Natural products in drug discovery and modern medicine:**

For thousands of years, natural products played an important role throughout the world in treating and preventing human diseases. Natural product medicines come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates. [58] The value of natural products in this regard can be assessed using 3 criteria:

(1) The rate of introduction of new chemical entities of wide structural diversity, and it serves as templates for semi synthetic and total synthetic modification,

(2) The number of diseases treated or prevented by these substances,

(3) The frequency of use in the treatment of disease.

An analysis of the drugs developed between 1981 and 2002 showed that natural products or natural product-derived drugs comprised 28% of all new chemical entities (NCEs) launched into the market [59]. In addition, 24% of these NCEs were synthetic or natural mimic compounds, based on the study of pharmacophores [58] related to natural products. This result suggests that natural products are important sources for new drugs and good lead compounds suitable for further modification during drug development. Since secondary metabolites from natural sources have been elaborated within living systems, they are often perceived as showing more "drug-likeness and biological friendliness than totally synthetic molecules," making them good candidates for further drug development [60].

Of these natural product-based drugs, paclitaxel (ranked at 25 in 2000), a plant-derived anticancer drug, had sales of $1.6 billion in 2000. The sales of 2 categories of plant-derived cancer chemotherapeutic agents were responsible for approximately one third of the total anticancer drug sales worldwide, or just under $3 billion dollars in 2002 [61, 62]; namely,

the taxanes, paclitaxel and docetaxel, and the camptothecin derivatives, irinotecan and topotecan.

**Approaches towards Evaluation of Medicinal Plants prior to Clinical Trials**

The requirements of health authorities on quality, safety and efficacy are based on the development procedure for the herbal as well as synthetic drugs. Health authorities are unwilling to accept traditional drug preparations from other cultural areas without well-documented data on quality, safety and efficacy. In many developing countries, appropriate utilization of local resources to cover drug needs is dependent on preliminary scientific study to determine the efficacy and safety of the preparations based on plant drugs that are used on an empirical basis in traditional medicine [63].

The phytotherapy acts as a bridge between traditional medicine and modern medicine. The development of plant derived drugs has always been a multi-step procedure starting with a crude extract followed by the standardized extract and ending up with isolated constituents. Quite often sufficient quality control and drug standardization is lacking for traditional recipes. Ethno pharmacological leads have resulted in the introduction of new single molecule drugs but have a greater role to play if crude extracts are accepted for clinical use in the West.

Clinical studies must be adapted to deal with the specifics of herbal Medicines in some cases. The number of patients required for undertaking clinical trial of medicinal plants is large not only since the study design needs to be adequate and statistically appropriate but also to provide to the control, cofounders and placebo groups to provide sufficient evidence for judging efficacy of the plant under study. The increase in patient number also increases the time commitment and the expenses involved. Therefore only a limited number of plants

can be subjected to clinical trials. Hence, it is essential to undertake appropriate preclinical testing to short list plants for clinical evaluation.

**Natural products as pharmacological tools**

There are many historical examples in which the natural product has not just been the medicinal product but has also helped reveal a novel aspect of physiology. For example, digitalis from foxglove showed the role of sodium-potassium-ATPase; morphine pointed the way to the receptors affected by endogenous opioids; muscarine, nicotine and tubocurarine helped to explore the different types of acetylcholine receptors, and so on [1, 10]. More recently, there has been interest in systematic searching for small-molecule inhibitors of key steps in biochemical processes (chemical genetics) [58]. Given that many assays involve identifying phenotypic changes in living cells (as opposed to binding interactions with isolated proteins), it is probable that natural products will provide useful probes for such studies [6, 10]. Moving beyond observations of phenotypic changes to defining the alterations in gene expression or protein function that are responsible will require advances in transcriptomic [59] and proteomic [60] methods.

**Pharmacovigilance of herbal medicines [64]:**

Currently, a majority of the adverse events related to the use of herbal products and herbal medicines that are reported are attributable either to poor product quality or to improper use. Member States of the World Health Organization (WHO) are therefore encouraged to strengthen national regulation, registration and quality assurance and control of herbal medicines. In addition, the national health authorities should give greater attention to consumer education and to qualified practice in the provision of herbal medicines. There is need to develop pharmacovigilance practices for herbal medicines. The current model of

pharmacovigilance has been developed in relation to synthetic drugs, and applying these methods to monitoring the safety of herbal medicines. Several tools used in pharmacovigilance of conventional medicines, such as prescription-event monitoring, and the use of computerized health-record databases, currently are of no use for evaluating the safety of herbal and other non-prescription medicines. Proposed European Union legislation for traditional herbal medicinal products will require manufacturers of products registered under new national schemes to comply with regulatory provisions on pharmacovigilance. In the longer term, other improvements in safety monitoring of herbal medicines may include modifications to existing methodology, patient reporting and greater consideration of pharmacogenetics and pharmacogenomics in optimising the safety of herbal medicines [65].

**Integration of *in silico* screening and natural products**

Facilities for high-throughput screening are now available in academic labs as well as in drug companies; however, the cost of random screening of very large collections of compounds can be prohibitive, and it makes sense to use *in silico* or virtual screening where possible to filter down the number of compounds used in real screens [58]. Whereas the Dictionary of Natural Products gives structural information on nearly 150,000 different compounds that could be used in virtual screening, the compounds would still have to be physically available for any predicted activity to be confirmed through testing in a relevant assay. As mentioned above, clustering of chemically related scaffolds can be very useful in guiding the synthesis of new compounds, but obviously there is a delay and expense in the synthesis. In an attempt to combine the advantages of virtual screening of chemically diverse natural products and their synthetic analogues with the rapid availability of physical samples for testing, an academic collaboration has established the Drug Discovery Portal .This brings

together a wide variety of compounds from academic laboratories in many different institutions in a database that can be used for virtual screening. When hits are predicted from the *in silico* screening, they can be sourced from the originating chemist for confirmatory tests. Often, there is an immediate link to expertise for the preparation of analogues to help start a lead optimisation programme [64].

**Conclusions**

Despite a period in which pharmaceutical companies cut back on their use of natural products in drug discovery, there are many promising drug candidates in the current development pipeline that are of natural origin. Technical drawbacks associated with natural product research have been lessened, and there are better opportunities to explore the biological activity of previously inaccessible sources of natural products. With the increasing acceptance that the chemical diversity of natural products is well suited to provide the core scaffolds for future drugs, there will be further developments in the use of novel natural products and chemical libraries based on natural products in drug discovery campaigns.

**Reason for selection of *Dalbergia sissoo*:**

A wide variety of plants possess pancreatic lipase inhibitory effects, including *Panax japonicus, Platycodi radix, Salacia reticulata, Nelumbo nucifera*, and so on. These pancreatic lipase inhibitory phytochemicals include mainly saponins, polyphenols and flavonoids. Several carbohydrates also possess pancreatic lipase inhibitory effects. Some of the most widely-studied materials among the many natural sources of pancreatic lipase inhibitors are the different types of tea (e.g. green, oolong, and black tea). A significantly different type of polyphenols (e.g. L- epicatechin) isolated from tea leaves, showed strong inhibitory activity against pancreatic lipase [66].

According to Indian herbal medicines, in Ayurveda system *Dalbergia sissoo* Roxb. (Charaka, Sushruta) were prescribed in obesit [67]. Herbal constituents and nutrient chemicals that have been proven effective includes various isoflavones, luteolin, genistein, apigenin, ponicidin and oridinin from *Rabdosia rubescens* and Ginseng, polysaccharo peptides in *Coriolus versicolor* (Turkeytail mushroom), and poly acetylenes in *Bidens pilosa*, as well as the much studied baicalein, berberine, epicatechin, and acteoside in the chinese herbs berberis, coptis chinensis, and phellodendron (Huang lian, Huang qin, and Huang bai), as well as *Epimedium sagitatum* (Yin yang huo), *Trichosanthes kirilowii* (Guo lou and Tian hua fen), and *Dalbergia odorifera* (Yin du huang tan). These herbs are found in various Chinese herbal formulations that often are used to treat endometriosis, obesity, uterine fibroids and ovarian cysts.

**METABOLOMICS**[254 &255]

Plant possesses an estimated value of 200,000 metabolites with different wonderful properties to increase our curiosity. Nature evolved this metabolite by the million years of hard work and screening so they are the fittest candidate on Darwin principles. (That's why most of the drugs are simply natural compounds or their Analogs.)

**Metabolomics**, which is the separation, detection and quantification of 'all' metabolites in a sample using either gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR), has been applied to many areas of plant sciences.

**Metabolomics** is a term used to describe the emerging science of measurement and analysis of metabolites, such as sugars and fats, in the cells of organisms at specific times and under specific conditions. The field of metabolomics overlaps with chemistry,

mathematics, genomics, transcriptomics, proteomics, computer science and statistics to understand the biology. (Fig 1.4)

The demands of a world where the human population continues to grow exponentially, combined with the impacts of global climate change and a finite fossil fuel resource, will place enormous demands on agricultural and forestry production systems. Delivering health outcomes through enhanced food quality (functional foods) will also lead to a better quality of life, as well as impacting positively on the health budgets of the developing world economies as the diseases of atherosclerosis, obesity and diabetes are directly related to the quantity and quality of the food we eat. All these developments will involve the need to fundamentally alter plant metabolism and tailor it for specific outcomes. Metabolites are at the heart of this process, yet our understanding of how metabolic pathways are regulated is at best rudimentary. The past few years have seen dramatic developments in high-throughput metabolite analysis (metabolomics), which, together with further advances that allow for cellular and subcellular resolution of metabolite analyses and the integration of these datasets with the other '-omics' through bioinformatics, make us ideally placed to make significant inroads into understanding these processes and their regulation in plants, thereby enabling rational design of novel herbal drug.

# CHAPTER II

# REVIEW OF LITERATURE

The literature review provides the background for understanding current knowledge on a topic and illuminates the significance for the new study. Thus one of the objectives of this literature review is to investigate the present state of the species and studies conducted in different countries and published over the past years.

## PHARMACOGNSY

**Banerjee K *et al.*, (1996)** documented the Morphology, germination behaviour and viability of conidia of powdery mildew of *Dalbergia sissoo.* The powdery mildew Symptoms of *Dalbergia sissoo* caused by *Phyllactinia dalbergiae* appeared only in the lower surface of the leaves [67].

**Kalia *et al.*, (1996)** have studied the *Euproctis subnotata* walk. a new pest of *Dalbergia sissoo* Roxb. [68]

**Minhas PS *et al.*, (1997)** studied the effect of saline irrigation and its schedules on growth, biomass production and water use of *Acacia nilotica* and *Dalbergia sissoo* in a highly calcareous soil [69].

**Singh *et al.*, (1997)** documented the effects of de-oiled tree seed cakes on growth and biomass production in *Dalbergia sissoo* seedlings. The study suggested that de-oiled tree seed cakes can be used as a potential, effective, cheaper and non-polluting organic source of nitrogen and other growth promoting substances [70].

**Rawat JS *et al.*, (1998)** have studied the influence of salinity on growth, biomass production and photosynthesis of *Eucalyptus camaldulensis* dehnh and *Dalbergia sissoo* Roxb seedlings. This study indicated that, no distinct relationship between leaf photosynthetic rate and dry-matter production was found. Thus study also indicated that low salt concentrations generally stimulated growth and biomass production [71].

**Meshram PB *et al.*, (1999)** have screened the *Dalbergia sissoo* seedlings from different seed sources for resistance to defoliator *Plecoptera reflexa* gue (Lepidoptera: Noctuidae). The seedlings from nine different seed sources were screened against defoliator, *Plecoptera reflexa* gue. It observed that seed source from Kanpur (U.P.) origin exhibited maximum resistance closely followed by Khoshala (Orissa). The performance of this origin was relatively better in all five parameters viz. damaged seedlings, leaves and leaf area consumed, larval population and chemical analysis (Polyphenol, protein, phosphorus, calcium and potassium) [72].

**Gera Mohit *et al.*, (1999)** documented the seed source variation in germination and early growth among ten indigenous populations of *Dalbergia sissoo* Roxb. Ten provenances /seed sources of *Dalbergia sissoo* Roxb scattered over a vide range of its natural occurrence were studied for germination, nursery and early field performance. Significant variations among the provenances were observed in parameters, viz., germination percentage, seedling height and collar diameter, and field height and survival percentage [73].

**Uniyal Poornima and Pokhriyal TC (2000)** have studied the effect of nitrogen treatment and seasonal variation on biomass production in *Dalbergia sissoo* seedlings. Effect of four different doses of nitrogen treatments i.e., 0, 50, 100 and 200 Kg N / hectare and

seasonal variations on biomass production was studied in *Dalbergia sissoo* seedlings in pots. An increase of biomass of leaf, stem and root were observed when compared to control [74].

**Newaj R *et al.*, (2001)** studied the effect of management practices on rooting pattern of *Dalbergia sissoo* under agri-silvicultural system. A field study was initiated at Jhansi during 1994 on different root management practices (deep ploughing, root barrier-polythene sheet, deep basin, pruning up to 40% height and control) on rooting pattern of *Dalbergia sissoo* Roxb, under agri- silvicultural system [75].

**Sah SP *et al.*, (2002)** documented the nutrient status of natural and healthy sissoo forest and declining plantation sissoo forest (*Dalbergia sissoo*, Roxb.) in Nepal. The water logging of soil was the main factor responsible for the decline of plantation sissoo forest [76].

**Shukla AN (2002)** documented the mortality of *Dalbergia sissoo* in India. The possible reasons are discussed with particular reference to *Fusarium solani* (Mart.) which is a secondary parasite on the dead roots and collaborative organism for the wilting of trees [77].

**Habib Rehman *et al.*, (2003)** have studied the kinetics of lead ions adsorption on sawdust of *Dalbergia sissoo* from aqueous solution. Adsorption of lead ions on sawdust of *Dalbergia sissoo* (Shisham) from aqueous solution was made under varying conditions of time and temperature. It was observed that amount of lead ions adsorbed increases with rise in temperature. The lead ions adsorption process obeys first order rate law, with activation energy of about 9.272 kJ $mol^{-1}$ [78].

**Mishra A *et al.*, (2003)** have documented the soil rehabilitation through afforestation by the evaluation of the performance of *Prosopis juliflora*, *Dalbergia sissoo* and plantations in a sodic environment [79].

**Gera Mohit *et al.*, (2004)** have studied the seed source variation as observed under scanning electron microscope in leaf characters of *Dalbergia sissoo* Roxb. Twenty seed sources of *Dalbergia sissoo* Roxb. were scattered over a wide range of its occurrence in India were studied for the pattern of variation in micro leaf characters such as upper stomatal frequency, lower stomatal frequency, upper stomatal size and lower stomatal size under scanning electron microscope (SEM). This shows the thickness of cuticle which contributed to the control of water loss from the underlying cells [80].

**Singh G and Bhati M (2005)** have studied the growth of *Dalbergia sissoo* in desert regions of western India using municipal effluent and the subsequent changes in soil and plant chemistry [81].

**Tewari VP and Kumar VSK (2005)** have reported the growth and yield functions for *Dalbergia sissoo* plantations in the hot desert of India grown under irrigated conditions [82].

**Rawat RS *et al.*, (2008)** have documented the inter-clonal variation in *Dalbergia sissoo* Roxb with respect to photosynthetic rate, transpiration rate and stomatal conductance in different climatic zones [83].

**Shi Lei *et al.*, (2008)** have demonstrated the study on anatomical structure variation and chemical properties of introduced *Dalbergia sissoo* Roxb. [84]

**Adenusi Adedotun A and Odaibo Alexander B (2009)** have reported the effects of varying concentrations of the crude aqueous and ethanolic extracts of *Dalbergia Sissoo* plant parts on *Biomphalaria Pfeifferi* egg masses [85].

**Bisht Rekha *et al.,* (2009)** have documented the effect of *Arbuscular mycorrhizal* fungi, *Pseudomonas fluorescens* and *Rhizobium leguminosarum* on the growth and nutrient status of *Dalbergia sissoo* Roxb. [86]

**PHYTOCHEMISTRY**

**FLOWER**

**Banerji A *et al.,* (1963)** have isolated the 7-methyltectorigenin, a new isoflavone from the flowers of *Dalbergia sissoo* [87].

**STEM-BARK**

**Mukerjee SK *et al.,* (1971)** have isolated the dalbergichromene – new neoflavonoid from stem-bark and heartwood of *Dalbergia sissoo* [88].

**Sharma A et al., (1980)** have isolated the isocaviudin, a new isoflavone glucoside isolated from *Dalbergia sissoo* [89].

**Kumar PV *et al.,* (1996)** have documented the isolation of the constituents of *Dalbergia sissoo* and their derivatives such as dalbergiphenol, dalbergiquinone, dalbergichromene, dalbergin, isodalbergin, methyl dalbergin, and melannein-along with an unidentified ester of dalbergiphenol with a higher fatty acid were isolated. Melannein has been isolated for the first time from *Dalbergia sissoo* [90].

**Farag SF *et al.,* (2001)** isolated the isoflavonoid glycosides from *Dalbergia sissoo*. Two isoflavone glycosides, biochanin A 7-O-[beta -D-apiofuranosyl-(1-->5)-beta -D-apiofuranosyl-(1-->6)-beta -D-glucopyranoside] and tectorigenin 7-O-[beta -D-apiofuranosyl-(1-->6)-beta -D-glucopyranoside], were isolated from *Dalbergia sissoo*. Their structures were elucidated on the basis of spectral and chemical evidence [91].

**Reddy Ramireddy Narahari *et al.*, (2008)** have isolated the O-Prenylated flavonoids from *Dalbergia sissoo*. A chalcone, 2,3-dimethoxy-4'-gamma,gamma-dimethylallyloxy-2'-hydroxychalcone and an isoflavone, 7-gamma,gamma-dimethylallyloxy-5-hydroxy-4'-methoxyisoflavone together with a known flavone, 7-hydroxy-6-rnethoxyflavone a known isoflavone, biochanin A and a known rotenoid, dehydroamorphigenin were isolated from the root bark of *Dalbergia sissoo*. The structures of these compounds were elucidated on the basis of spectral and chemical studies [92].

**LEAF**

**Rana Vikas *et al.*, (2009)** have reported the structure of the polysaccharides isolated from leaves of *Dalbergia sissoo* Roxb [93].

**PHARMACOLOGY**

**ARIEAL PARTS**

**Sarg T *et al.*, (1999)** documented the phytochemical and pharmacological studies of *Dalbergia sissoo* growing in Egypt. The isoflavones irisolidone, biochanin-A, muningin, tectorigenin, prunetin, genestein, sissotrin and prunetin-4-O-galactoside, the flavone nor-artocarpotin, and beta-amyrin, beta-sitosterol and stigmasterol were isolated and identified from the green branches of aerial parts of *Dalbergia sissoo* Roxb using silica gel column chromatography and spectral analysis. The alcohol extract showed a dose-dependent inhibitory effect an the motility of isolated rabbit duodenum, pronounced bronchodilation, as well as significant anti-inflammatory, antipyretic, analgesic, and estrogen-like activities [94].

**HEART WOOD**

**Ramakrishna NVS *et al.*, (2001)** have documented the screening of natural products for new leads as inhibitors of beta-amyloid production: Latifolin from *Dalbergia sissoo*.

Latifolin isolated from the methylene chloride extract of the heartwood of *Dalbergia sissoo* and found to exhibit the inhibition of beta-amyloid synthesis with an $IC_{50}$ of 180µg [95].

**Shrestha Suraj Prakash *et al.*, (2008)** have documented the nitric oxide production inhibitory activity of flavonoids present in trunk exudates of *Dalbergia sissoo*. From the Methanolic extracts of trunk exudates of *Dalbergia sissoo* yielded 26 known compounds. The ability of the isolated compounds to prevent nitric oxide (NO) production by LPS-stimulated J774.1 cells was also studied. All of the isolated compounds except, formononetin, and zenognosin B exhibited significant activity in a concentration-dependent manner [96].

**Yadav H *et al.*, (2008)** have studied the antimicrobial property of a herbal preparation containing *Dalbergia sissoo* and *Datubra stramonium* with cow urine against pathogenic bacteria. The anti bacterial activity for gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) and gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) bacteria were studied. Antibacterial activity was compared to standard antibiotic drugs i.e. Chloramphenicol (30 mcg), Ampicillin (10 mcg), Nalidixic acid (10 mcg) and Rifampicin (30 mcg). Cow urine extract was found to be most active against both gram-positive as well as gram-negative bacteria [97].

**Roy Nayan *et al.*, (2011)** have reported a detailed study on the antioxidant activity of the stem bark of *Dalbergia sissoo* Roxb an Indian medicinal plant. Aqueous and methanolic extracts (AED and MED respectively) of the stem bark of the plant, was evaluated for the antioxidant activity by in vitro chemical analyses involving the assays of (1) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (2) ferric ion reducing power (3) ferrous ion chelating activity and (4) Au nanoparticle formation potential [98].

**LEAVES**

**Meshram PB (2000)** studied the antifeedant and insecticidal activity of *Dalbergia sissoo* against defoliator *Plecoptera reflexa* Gue. (Lepidoptera: Noctuidae). Crude extracts of fresh leaves of 14 different plants were tested against third instar larvae of defoliator, *Plecoptera reflexa* to evaluate their antifeedant and insecticidal activities [99].

**Hajare SW *et al.*, (2000)** reported the analgesic and antipyretic activities of *Dalbergia sissoo* leaves. The alcoholic extract of *Dalbergia sissoo* leaves was studied using acetic acid-induced writhing in mice and by Randall-Selitto assay. The central analgesic activity of SLE was studied using hot-plate method and tail-clip test in mice. The extract (1000 mg/kg) significantly increased the pain threshold capacity in rats in Randall-Selitto assay and the reaction time in hot-plate test but not in tail-clip test. It also showed significant antipyretic activity in Brewer's yeast-induced pyrexia in rats throughout the observation period of 6 h [100].

**Ansari MA *et al.*, (2000)** have studied the larvicidal and repellent actions of the leaves of *Dalbergia sissoo* Roxb. (F. Leguminosae) oil against mosquitoes. This study was carried out against *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* under laboratory conditions. The oil showed repellent action when 1 ml of oil was applied on exposed parts of human volunteers. They were protected from mosquito bites for 8-11 h. The protection (91.6 +- 2%) obtained with sissoo oil was comparable to that with commercial Mylol oil (93.8 +- 1.2%) consisting of di-butyl and dimethyl phthalates [101].

**Hajare SW *et al.*, (2001)** documented the anti-inflammatory activity of *Dalbergia sissoo* leaves. Ethanolic extract 90% of the plant was studied in different models of

inflammation in rats after oral administration at doses of 100, 300 and 1000 mg/kg. It inhibited carrageenin, kaolin and nystatin-induced paw oedema, as well as the weight of granuloma induced by a cotton pellet. In acute toxicity studies, the extract was found to be safe up to 10.125 g/kg, p.o. in the rat. It was concluded that the D. sissoo leaf extract possessed significant anti-inflammatory activity (in acute, sub-acute and chronic models of inflammation) without any side effect on gastric mucosa [102].

**Brijesh S *et al.*, (2006)** have reported the studies on *Dalbergia sissoo* (Roxb.) leaves for possible mechanism of action in infectious diarrhea. Antibacterial, antiprotozoal, and antiviral activities of the plant decoction were checked by agar dilution method, tube dilution method, and neutral red uptake assay. Cholera toxin (CT) and *Escherichia coli* labile toxin (LT) were assayed by ganglioside monosialic acid receptor ELISA [103].

**Ragab Amany *et al.*, (2007)** have reported the biological evaluation and study of polysaccharides of *Dalbergia sissoo* Roxb growing in Egypt. The anti-tumor, anti-oxidant, and antimicrobial activities of *D.sissoo* extracts were also examined. Since the polysaccharides prepared from different organs (leaves 2.5%, stem 2.2%, bark 1.2%) possessed anti-inflammatory activities, they were subjected to further phytochemical studies, using paper chromatography and GC/MS analysis. The leaf polysaccharides consist mainly of rhamnose (77%) in addition to glucose (23%). The stem polysaccharide consists of rhamnose (47%), glycerol (46%) and galactose (7%). The bark polysaccharide consists of rhamnose (18%), fructose (2.5%), glucose (74.5%) and galactose (6%) [104].

## GENETICS

**Pradhan C *et al.*, (1998)** have documented the propagation of *Dalbergia sissoo* Roxb. through in vitro shoot proliferation from cotyledonary nodes. Multiple shoots were

induced from cotyledonary nodes derived from 1-week-old axenic seedlings on Murashige and Skoog's medium containing N-6-benzyladenine (BA), kinetin (Kn), isopentenyladenine (2iP) or thidiazuron (TDZ), with BA being the most effective growth regulator [105].

**Puri S *et al.,* (1999)** renowned the geographical variation in rooting ability of stem cuttings of *Azadirachta indica* and *Dalbergia sissoo*. This study showed that no significant variation in survival was evident with respect to provenance or auxin treatment [106].

**Gera Mohit *et al.,* (2000)** studied the preliminary observations on genetic variability and character association in *Dalbergia sissoo* Roxb. Twenty seed sources of *Dalbergia sissoo* Roxb. scattered over a wide range of its natural occurrence in India were studied for the pattern of genetic variation and character association after two and a half years of field planting in a statistically laid out trial. The results revealed the presence of highly significant variations among the provenances for height, number of branches and survival percentage [107].

**Joshi I *et al.,* (2003)** have documented the studies on effect of nutrient media for clonal propagation of superior phenotypes of *Dalbergia sissoo* Roxb through tissue culture. Two nutrient media MS and B5 were used to find out the suitability of the medium. Bud break was achieved in both of the media within 6-8 days under different media combinations supplemented with BAP (0.10-1.0 mg/l) alone as well as in combinations with IAA or NAA (0.10 to 0.50 mg/l). Maximum percentage of bud break (100%) was achieved in both of the media. Maximum number of shoots per explant (8.04) was observed in the MS medium supplemented with 1.0 mg/l BAP + 0.25 mg/l NAA [108].

**Arif Mohd *et al.*, (2009)** have studied A Comparative Analysis of ISSR and RAPD Markers for Study of Genetic Diversity in Shisham (*Dalbergia sissoo*). Two DNA-based molecular marker techniques, intersimple sequence repeat (ISSR) and random amplified polymorphism DNA (RAPD) were compared to study the genetic diversity in this species [109].

**Ginwal HS and Maurya Shalini Singh (2010)** have reported the evaluation and optimization of DNA extraction method for *Dalbergia sissoo* leaf. The DNA was isolated using cetyl trimethyl ammonium bromide (CTAB) method. The yield was approximately 100 to 400 mcg DNA per 100 mg of leaf tissue. The genomic DNA obtained by this method was suitable to be used in RAPD and ISSR analysis. This extraction method would allow the molecular analysis of DNA from different clones of *D. sissoo* [110].

**Other species**

**Souza Brito A.R.M *et al.*, (1998)** have studied the gastric antiulcerogenic effects of *Dalbergia monetaria* L. in rats. The antiulcerogenic activity of lyophilized aqueous extract (LAE) of the plant was studied in four models of gastric ulcers in rats. LAE showed a dose dependent inhibition of gastric lesions induced by indomethacin, ethanol, pylorous ligature and hypothermic-restraint stress. LAE extract was more effective against hypothermic-restraint stress-induced lesions and less effective against indomethacin-induced gastric mucosal damage [111].

**Geoffrey Kite C *et al.*,** (2010) have isolated the dalnigrin, a neoflavonoid marker for the identification of Brazilian rosewood (*Dalbergia nigra*) in CITES enforcement [112].

**Kamal Al-Khalifa F (2006)** had documented the propagation of (*Dalbergia melanoxylon*). Seeds were subjected to different pregermination treatments. Vegetative propagation by shoot cuttings was investigated using Indole Buteric acid (IBA) and Naphthaline acetic acid (NAA). The results showed that sulphuric acid was lethal to the embryo. Seed germination was highest for seeds treated with hot water, cold water for one day or without treatment, where no significant differences found among them [113].

**Donnelly DMX *et al.*, (2001)** have isolated the neoflavanoids of *Dalbergia cultrate*. The known neoflavanoids (S)-4-methoxydalbergione, dalbergin, and stevenin, the heartwood of *Dalbergia cultrata* Grah contains a new 3,3-diphenylprop-l-ene (Ia) [114].

**Mrudula Kale *et al.*, (2007)** have studied the anti-inflammatory activity of ethanolic extract of bark of *Dalbergia lanceolaria* in mice and rats. The ethanol extract was studied in albino mice using TPA-, EPP- and AA-induced ear edema models. The extract also showed significant activity against turpentine-induced exudative changes and no activity against granular tissue formation in cotton pellet-induced granuloma in albino rats [115].

**Cheng ZJ *et al.*, (1998)** studied the antioxidant properties of butein isolated from *Dalbergia odorifera* [116].

# CHAPTER III

## AIM AND SCOPE OF THE STUDY

Plant possesses an estimated value of 200,000 metabolites with different niche properties to increase our curiosity. The ethnomedical information of the plant reveals that the leaves of *Dalbergia sissoo* roxb. was used as eye ailments, abortifacient, anthelmintic, antipyretic, aphrodisiac, expectorant, refrigerant, stomach problems, and syphilis. According to Ayurveda system of medicine, leaves of this plant posseses anti obesity property. In Chinese medicine *Dalbergia odorifera* is used for anti obesity treatment. The purpose is to link the traditional concepts and uses of herbal drugs, herbal products and certain phytochemicals for potential phytomedicine using modern scientific approaches.

The phytochemical studies on the leaves have been reported for the presence of flavanoids, tannins, glycosides, sterols and carbohydrates. Some phytochemical studies have been documented for the isolation of flavonoids and anthraquinone glycosides in this plant. Anti-obesity has become an important issue for food and drug research in which natural products has been intensively researched for this purpose. Therefore, the present research is focussed to investigate the anti-obesity effects of *Dalbergia sissoo*.

Hence this work has been designed in such a way to carry out the following studies on the leaves *Dalbergia sissoo.*

- Pharmacognostical studies on the leaves.
- Induction of callus culture from the leaf by tissue culture for high concentration of secondary metabolite production which is to be ascertained by qualitative and quantitative analysis of phytochemical analysis.

- Preliminary phytochemical screening on the extracts of *Dalbergia sissoo*.
- Estimation of total phenols, total tannins and total flavanoids.
- Isolation of active principle from ethanolic extract of *D.sissoo* using column chromatography.
- To study the analytical processes such as spectroscopy (UV, FTIR, MS & NMR) and chromatographic techniques (TLC) for structural elucidation of the active principle.
- Screening of the ethanolic extract and isolated active principle from the leaves for following pharmacological activities.
    - Invitro antioxidants activity
        - DPPH radical scavenging activity
        - Hydrogen peroxide scavenging activity
        - Total Anti oxidant assay
- To study the detailed effects of extract and active principles isolated from *D.sissoo* for anti obesity activity using
    - Invitro Chicken pancreatic lipase inhibition assay
    - Evaluation of the acute toxic effects of *Dalbergia sissoo* in Zebra fish (*Danio rerio*) embryos by fish embryo toxicity test.
    - Anti obesity and Lipid lowering effect of *Dalbergia sissoo* using Zebrafish (*Danio rerio*)
- To study the molecular mechanism and active site file modelling of our phytomedicine using Bioinformatics tools like docking studies (GOLD/AutoDock) for the rationale herbal drug designing.

# CHAPTER IV

# PHARMACOGNOSTICAL STUDIES

***Dalbergia sissoo* Roxb.**

**Family**: Fabaceae.

## SECTION A

## GENERAL DESCRIPTION OF THE PLANT

### SYSTEMATIC POSITION [117-119]:

| | | |
|---|---|---|
| Kingdom | : | Plantae |
| Subkingdom | : | Angiosperms |
| Super division | : | Eudicots |
| Division | : | Magnoliophyta |
| Class | : | Magnoliopsida |
| Order | : | Fabales |
| Family | : | Fabaceae |
| Subfamily | : | Faboideae/ Papilionoideae |
| Genus | : | *Dalbergia* |
| Species | : | *sissoo* |

### SYNONYM

*Amerimnon sissoo, Dalbergia pseudo-sissoo*

### COMMEN NAME

Sissoo, sisu, sheesham, tahli and sometimes referred to as Indian Rosewood.

### VERNACULAR NAME

English : India teakwood, Indian dalbergia, Indian rosewood

Hindi : Shisham, sissu, sissai

Kanada : Agaru

Malayalam : Iruvil

Sanskrit : Shinshapa, aguru

Tamil : sisu itti, gette, nukku kattai

Telugu : Errasissu

**GEOGRAPHICAL DISTRIBUTION**

It is found in Pakistan, Oman, Bhutan, India, Nepal, Myanmar, Iran, Afghanistan, Bangladesh, and Malaysia.

**HABIT AND HABITAT OF PLANT**

*Dalbergia sissoo* is found in tropical to subtropical climates in natural and planted forests. It mainly grows along forest margins near streams and rivers, hammocks, canopy gaps, agricultural areas, disturbed sites and roadsides. It often occurs in association with *Acacia catechu.*

It survives in areas with a mean annual rainfall of 500-4500mm and often associated with seasonal monsoon and periods of drought up to six months (Fig 2.1). The temperature hardiness is from slightly below freezing to 50 degree Celsius and can grow from altitudes ranging at sea-level to 1500 meters .It grows best in porous well-drained soils like sands, sandy loams, gravels, and alluvial soils, but does poorly in heavy clay and waterlogged soils. The pH ranges from 5-7.7and the species has a low salt tolerance.

**DESCRIPTION OF THE PLANT:**

**LEAVES**

The leaves are alternately arranged, compound and oddly pinnate with 3-5 glabrous, leathery leaflets, elliptical to ovate, tapering to a point and 2.5-3.6cm in diameter (Fig 2.3).

**FRUIT**

Fruits are indehiscent, 5-7.5cm long and 8-13mm wide, rounded with minute points, pale brown in color, and persistent on the tree. The fruit is a light brown indehiscent pod, 5-9 cm long, 10-12 mm wide, thin and glabrous and with conspicuous veins. There are 1-5 seeds/pod (Fig 2.4 & 2.5).

**SEED**

The seed is kidney-shaped, thin, flat, and light brown with 1-4 seeds in a pod, kidney-shaped, 8-10 mm long, 4-5.5 mm wide, pale brown to almost black, flat and with thin testa. There are 40000-55000 seeds per kg (Fig 2.5).

**FLOWER**

Flowers are sessile, arranged in axillary panicles, 2.5-3.7cm long, inconspicuous, white to dull yellow. Flowers are fragrant, with pubescent sepals 4-5mm long, and petals 6-8mm long (Fig 2.6 & 2.7).

**WOOD**

It is brown, hard and heavy close grained. It appears smooth and good polish (Fig 2.8).

**STEM**

Young shoots downy, drooping; established stems with light brown to dark gray bark to 2.5 cm thick, shed in narrow strips; large upper branches support a spreading crown.

**ROOT**

A long taproot and numerous surface roots which produce suckers

## SECTION- B

## MICROSCOPICAL STUDIES OF THE LEAVES

### MATERIALS AND METHODS [120-126]

Plants were collected from Madurai and identified by Dr. Sasikala Ethirajulu, Botanist, Siddha Central Research Institute, Chennai – 106.

Petiole and leaf were fixed in FAA solution (70% ethyl alcohol, formalin and acetic acid in the ratio of 90 mL: 5 mL: 5 mL). The materials were left in the fluid for three days, after which they were washed in water and dehydrated with tertiary butyl alcohol. Paraffin wax was filtered and the specimens were embedded in wax for sectioning.

Transverse sections of petiole and leaf were taken using microtome and stained with toluidine blue. All sides, after staining in toluidine blue were dehydrated by employing graded series of ethyl alcohol (70 %, 90%, 100% alcohol) and xylol-alcohol (50-50) and passed through xylol and mounted in DPX mountant (Johansen 1940).

Clearing of leaves for studying stomatal number and stomatal index was done by using 5% sodium hydroxide along with chlorinated soda solution supplemented with gentle heat. Quantitative microscopy was carried out and values were determined as per the procedure given in Wallis (1997). Photomicrographs were taken with the help of Nikon Eclipse E200 Microscope.

**Microscopic:**

**Petiole:** Transverse section of petiole is circular in outline (Fig 3.1). The outer most epidermis is made up of single layer of cells. Most of the cells elongate to form uniseriate trichome. Epidermal cells are papillose. The cortex is broad and composed of round, closely arranged parenchyma cells (Fig 3.2 & 3.3). In the centre 'U' shaped with strongly incurved

ends and approximately circular, leaving a small gap on the adaxial side, large, collateral vascular bundle is seen. The vascular bundle is surrounded by sclerenchyma fibres (Fig 3.4).

**Leaflet:**

**Midrib:** Transverse section of midrib shows a flat surface on the adaxial side and convexity on the abaxial side (Fig 3.5). The epidermis is made-up of single layer of rectangular transversely elongated cells (Fig 3.6). The abaxial epidermis is papillose and inner walls are gelatinized. The hypodermal region of adaxial and abaxial epidermis is composed of 2 to 4 rows of collenchyma cells.

A large arc shaped collateral vascular bundle is situated in the centre. Sclerenchyma fibres are present on the adaxial and abaxial side of the vascular bundles.

**Lamina:** Leaf is dorsiventral in structure (Fig 3.7). The adaxial epidermal cells are larger than the abaxial epidermal cells. Hypodermis on the upper side is made up of large rectangular parenchyma cells (Fig 3.7). The palisade tissue is made up of 2 rows of columnar closely packed cells. The spongy tissue is composed of 5 to 7 rows of loosely arranged round parenchyma cells (Fig 3.10). A small crystalline grains or prisms or rod shaped crystals are seen in the mesophyll tissue. The stomatal index for abaxial epidermis is 17 to 21; palisade ratio is 3 to 4; vein islet number ranges from18 to 22 (Fig 3.8 & 3.9). The smaller veins of the leaf are vertically transcurrent.

**Epidermis in surface view**

The adaxial foliar epidermis is made up of polygonal parenchyma cells with straight wall and devoid of stomata. Uniseriate trichomes are noticed (Fig 3.7).

The abaxial foliar epidermal cells are also polygonal in shape with straight walls but smaller in size. It is perforated by rubiaceous stomata or stomata surrounded by a rosette of cells (Fig 3.8 & 3.9).

**Trichome**

Trichomes are numerous, simple, uniseriate with a short basal cell accompanied by an elongated terminal cells with blunt tip (Fig 3.7).

## SECTION C

## POWDER MICROSCOPY

### ORGANOLEPTIC CHARACTERS:

Nature: Coarse

Color: Greenish yellow

Odour: Characteristic

Taste: Bitter followed by astringent taste.

Powder microscopy of the leaves showed the following characters,

- Epidermal cells with rubiaceous stomata.
- Uniseriate trichomes are noticed
- Polygonal parenchyma cells are present
- Sclerenchyma fibres are present
- Vascular bundles are seen
- Lignified xylem fibres are abundant in the powder

## SECTION-D

## QUANTITATIVE MICROSCOPY

This is useful for identification, characterization, and standardization of crude drugs. A clear idea about the identity and characteristic features of the drug can be obtained after several numbers of determinations; the characteristic's number obtained is noted and compared with a standard value to find out whether it is within the range and standard deviation.

### STOMATAL NUMBER AND STOMATAL INDEX [122-126]

**Stomatal number**: The average number of stomata present in 1 square millimeter area of each surface of a leaf epidermis is termed as stomatal number [123].

**Stomatal index:** The stomatal index is the percentage of the number of stomata formed by the total number of epidermal cells including stomata, each stoma being counted as one cell.

**Determination of stomatal number and stomatal index**

To study the stomatal morphology (type of stomata), stomatal number and stomatal index of leaf, the leaf was subjected to epidermal peeling by partial maceration employing the Jeffrey's maceration fluid.

A fragment was transferred in to microscopic slide and the mount of lower and upper epidermis was prepared with a small drop of glycerol solution at one side of the cover slip to prevent the slide from drying. The slide was examined under 45X objective and 10X eye piece to which a microscopical apparatus was attached. Circle (O) like mark was marked on the drawing paper for each stoma. The average number of stomata/square mm for each surface of the leaf was calculated and their values are tabulated in table 1**.**

For stomatal index, the glycerin mounted leaf peeling as mentioned above was made and circle (O) like mark for each stomata and a cross (X) like mark for each epidermal cells was marked on the drawing paper. The stomatal index was calculated by using the formula,

**Stomatal index** $\quad S.I = \frac{S}{E+S} \times 100$

Where S = Number of stomata in 1 sq mm area of leaf and E = Number of epidermal cells (including trichomes) in the same area of leaf. The values are tabulated in table 1.

**VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER**

The term vein islet is used to denote the minute area of photo synthetic tissue encircled by the ultimate division of the conducting strands. The number of vein islets per square mm area is called vein- islet number.

Vein termination number may be defined as the number of vein terminations present in one square mm area of the photosynthetic tissue. [124]

**Determination of Vein Islets and Vein Terminations**

The fragment of leaf lamina with an area of not less than 1 sq mm excluding the midrib and the margin of the leaf was taken. The fragments of leaf lamina were cleared by heating in a test tube containing chloral hydrate solution on a boiling water bath until clear. The cleared fragments were stained with safranin solution and a temporary mount was prepared with glycerol solution. The stage micrometer placed on the microscopic stage, examined under 10X objective and 6X eye piece and an area of 1 sq mm square was drawn. The cleared leaf piece was placed on the microscope stage, the vein islets and vein terminals included in the square was drawn.

The number of vein islets and terminals within the square including those over lapping on two adjacent sides and excluding those intersected by others two sides were

counted. The results obtained in the number of vein islets and terminals in1 sq mm were tabulated in table 1.

**PALISADE RATIO**

Palisade ratio is the average number of palisade cells under one epidermal cell. It is another important criterion for identifications and evaluations for crude drugs. Since it is constant for a plant species which is useful to differentiate the species and does not altered based on geographical variation [124].

**Determination of Palisade Ratio**

Epidermal peeling was done by partial maceration by Jeffery's maceration fluid were prepared. A fragment was transferred into a microscopical slide and the mount of upper epidermis was prepared with a small drop of glycerol on one side of the cover slip to prevent the preparation from drying. The same was examined under 45X objective and 10X eye piece. Four adjacent epidermal cells were traced; focusing gently downward to bring the palisade cells into view and sufficient palisade cells to cover the outlined four epidermal cells were then traced. The palisade cells under the epidermal cells were counted and calculate the palisade ratio by using the following formula and the results were tabulated in table 1.

**Palisade ratio = Avg. number of palisade cells beneath the 4 epidermal cells/4**

## SECTION - E

## PHYSICAL PARAMETERS

### POWDER ANALYSIS

The behavior of the powder with different chemical reagents was carried out as per standard procedure [123]. The observations are presented in table 2.

### Fluorescence analysis

The fluorescent analysis of the drug powder as well as the plant extracts of *D.sissoo* were carried out and the observations are tabulated in tables 3 & 4**.**

### STANDARDIZATION PARAMETERS [126]

The evaluation of ash values, loss on drying, foreign organic matter and extractive values etc. gives a clear idea about the specific characteristics of crude drug under examination, besides its macro-morphological or cyto-morphological, microscopical nature in both its entire and its powder form. These diagnostic features enable the analyst to know the nature and characteristic of crude drugs and further evaluation of different parameters indicate their acceptability. The procedures recommended in Indian Pharmacopoeia, 1996 and WHO guidelines, 1998 were followed to calculate total ash, water-soluble ash, acid-insoluble ash and loss on drying. The percentages of extractive values for different solvents were also determined for this plant.

### Determination of Volatile Oil

Volatile oils are characterized by their odor, oil like appearance and also it has ability to volatilize at room temperature. Chemically they are mixtures of monoterpenes, sesquiterpenes and their oxygenated derivatives. Volatile oils can be estimated by hydro distillation method.

An accurately weighed 100g of plant material was crushed and introduced in to the flask containing distilled water until one third of the plant material was immersed and few pieces of porcelain bits were added. The flask containing liquid was heated until it boils. After 3h, heating was stopped and the collected oil was recorded on the graduated receiver tube. Oil content of the plant material was calculated in mL/100g of plant materials. The result is presented in table 5.

**Determination of foreign organic matter**

The part of organ or organs other than those specified in the definition or description of the crude drugs is defined as foreign organic matter.

An accurately weighed 100g of air dried coarse drug and spread out in a thin layer. The sample drug was inspected with the unaided eye or with the use of 6X lens and the foreign organic matter was separated manually as completely as possible and weighed. The percentage of foreign organic matter was calculated with reference to the weight of the drug taken. The result is presented in table 5.

**Determination of Moisture Content (Loss on Drying)**

An accurately weighed 10g of coarsely powdered drug was placed in a tared evaporating dish. Then the dish was dried at 105$^{o}$C for 5h and weighed. The drying and weighing was continued at one hour intervals until the difference between the two successive weighing is not more than 0.25%. The loss on drying was calculated with reference to the amount of powder taken. The readings are tabulated in table 5.

**Determination of Ash values** [126]

**Ash Content**

The residue remaining after incineration is the ash content of crude drug, which simply represents inorganic salts naturally occurring in the drug or adhering to it or deliberately added to it as a form of adulteration.

**Determination of Total Ash**

An accurately weighed 3g of air dried coarsely powdered drug was taken in a tarred silica crucible and incinerated at a temperature not exceeding 450$^{o}$C, until free from carbon then allowed to cool and weighed. The percentage of ash was calculated with reference to the air dried drug.

**Determination of Acid Insoluble Ash**

The total ash obtained from the previous procedure was mixed with 25mL of 2M hydrochloric acid and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450$^{o}$C, cooled in desiccators and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried drug.

**Determination of Water Soluble Ash**

The total ash obtained from the previous procedure was mixed with 25mL of water and boiled for 5min in a water bath, and then the insoluble matter was collected in an ashless filter paper and washed with hot water, dried and ignited for 15min at a temperature not exceeding 450$^{o}$C, cooled in desiccators and weighed. The insoluble matter was subtracted from the weight of the total ash; the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

The values in respect of the total ash values, acid insoluble ash, water soluble ash and water insoluble ash are tabulated in table 5.

**Determination of Extractive Values**

Extractive values used to determine the amount of active principle or phyto constituents present in the given amount of plant materials, when extracted with suitable solvents. Determination of extractable matter determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material and herbal formulation. The extraction of crude plant materials with suitable solvents yields a solution containing different phyto constituents. Composition of the phyto constituents in a particular solvent depends upon the nature of drugs and solvents used. This is used to provide preliminary information on the quality of particular sample.

**Determination of ethanol soluble extractive**

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of ethanol in a closed flask for 24h, shaking frequently during the first 6h and allowed to stand for 18h. Thereafter filtered rapidly, taking precautions against loss of ethanol. Then evaporate 25mL of the filtrate to dryness in a tarred flat bottomed shallow dish dry at 105$^{o}$C and weighed. The percentage of ethanol soluble extractive was calculated with reference to the air dried drug.

**Determination of water soluble extractive:**

An accurately weighed 5g of the air dried coarsely powdered drug was macerated with 100mL of chloroform water in a closed flask for 24h, shaking frequently during the first 6h and allowed to stand for 18h. Thereafter filtered rapidly, taking precautions against loss of chloroform water. Then evaporate 25mL of the filtrate to dryness in a tarred flat bottomed shallow dish dry at 105$^{o}$C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried drug.

**Determination of Hexane and petroleum ether soluble extractive:**

The procedure adopted under ethanol soluble extractive was followed using petroleum ether as a solvent instead of ethanol.

**Determination of ether soluble extractive:**

The procedure adopted under ethanol soluble extractive was followed using ether as a solvent instead of ethanol.

**Determination of methanol soluble extractive:**

The procedure adopted under ethanol soluble extractive was followed using methanol as a solvent instead of ethanol.

**Determination of chloroform soluble extractive:**

The procedure adopted under ethanol soluble extractive was followed using chloroform as a solvent.

The extractive values obtained for different solvents are presented in table 6**.**

**Determination of Foaming Index**

Plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of foaming index.

An accurately weighed 1g of the coarse plant material was transferred into a 500mL conical flask containing 100mL of boiling water. The flask was maintained at moderate boiling for 30min. The solution was cooled and filtered into a 100mL volumetric flask and sufficient distilled water was added to dilute to volume. The decoction was poured into 10 stoppered test tubes in successive portions of 1mL, 2mL, etc. upto 10mL, and the volume of the liquid in each tube was adjusted with water upto 10mL. The tubes were stoppered and

shaken in a length wise motion for 15sec (two shakes/sec) and allowed to stand for 15min. The height of foam was measured. If the height of the foam in every tube was less than 1cm the foaming index was less than 100. If a height of foam of 1cm was measured in any test tube, the volume of the plant material decoction in this tube (a) was used to determine the index. If this tube was first or second tube in a series, an intermediate dilution was prepared in a similar manner to obtain a more precise result.

If the height of the foam was more than 1cm in every tube, the foaming index was over 1000. In this case, the determination was repeated using a new series dilution of the decoction in order to obtain a result. The foaming index was calculated using the following formula 1000/A where A was the volume in mL of the decoction used for preparing the dilution in the tube where foaming to a height of 1cm was observed. The result obtained is presented in table 6.

**Determination of Swelling Index**

Swelling index is the volume in mL taken up by the swelling of 1g of plant material under specified conditions. Its determination is based on the addition of water. Using a glass stoppered measuring cylinder, the material is shaken repeatedly for 1h and then allowed to stand for required period of time. The volume of the mixture (in ml) is read.

About 1g of weighed powder was introduced in to a 25mL glass stoppered measuring cylinder, and 25mL of water was added and the mixture was shaken thoroughly every 10min for 1h and allowed to stand for 3h at room temperature. The volume in mL occupied by the plant material including the sticky mucilage was measured. The mean value of the individual determination was calculated related to 1gm of plant material and the result is tabulated in table 6.

# CHAPTER V

# CALLUS INDUCTION FROM *Dalbergia sissoo* Roxb.

## INTRODUCTION

### Tissue and Cell Culture [127-134]

Tissue culture is a technique of growing plant cells by culturing explant aseptically on a suitable nutrient medium.

### Callus culture

The culture of undifferentiated mass of cell on agar media produced from an explant of a seedling or other plant part is called callus culture. For callus formation, PGRs such as auxins and cytokinins, both are required. Callus can be subcultured indefinitely by transferring a small piece of the same to fresh agar medium. Subculturing needs to be done every 3-5 weeks in view of cell growth, nutrient depletion and medium drying.

## AIM AND OBJECTIVES OF THE STUDY [135]

*Dalbergia sissoo* (Fabaceae) is a fast growing tree and is widely planted. The bark, wood, root, leaf, juice and seed oil of the tree have many medicinal properties. *Dalbergia sissoo* is a folk remedy for stimulant, excoriations, and gonorrhea and skin ailments. Lack of disease free planting material and quality is greatly affecting the economy of many developing countries of the world.

Sissoo is mostly propagated by seeds or by planting suckers. But the techniques are laborious and time consuming as far as production of large number of homogeneous plants are concerned. Most of the crop plants especially those propagated by vegetative means are systematically infected with one or more pathogens. Furthermore, sissoo has been seriously affected in recent years by different fungal or insect pest causing diseases, such as die back,

root and butt rot, leaf defoliation, leaf rolling, foliage rust and powdery mildew. The exchange of plant propagation material from country to country or continent to continent involves the possible dissemination of these diseases. The conservation of *Dalbergia* spp. genetic resources are also problematic since the diseased free species are not possible to conserve in field gene bank where maintenance cost is also high. Somatic embryogenesis has been reported in *D. sissoo* from callus cultures derived from semi-mature zygotic embryos.

The production and analysis of secondary metabolites in culture have possible ecological and physiological functions so their significance in pharmacology must be very high. The major advantage of the cell cultures includes synthesis of bioactive secondary metabolites in controlled environment, independently from climate and soil conditions. The use of *in vitro* plant cell culture for the production of chemicals and pharmaceuticals has made advances in plant science. The *invitro* plant cell cultures have potential for the production of secondary metabolite. Our research is to design a rapid, inexpensive method to isolate high concentration and disease resistance secondary metabolite for obesity.

## MATERIALS AND METHODS

### Plant material [136-141]

The plant was healthy and free from symptoms of disease with show good biomass. The plant which was used for experiment is collected from Madurai. Leaves were collected from the plant and trimmed to 2-3 cm size for further work.

### Explant sterilization

For the surface sterilization, the explants were washed thoroughly in running tap water for 30 minutes. Next, the leaves were rinsed in a 10% bleach solution (1% sodium hypochlorite) with two drops of Tween 20 for ten minutes, and then rinsed three times at five

minutes each with sterile DDH2O. After these treatments explants were taken inside the laminar hood for further sterilization. Here 2-3 sterile water washings are given. After these washings, explants were taken out and dipped in 70% ethyl alcohol for 30 seconds. After alcohol dip, explants were surface sterilized with freshly prepared 0.1% w/v aqueous solution of Mercuric chloride for 5 minutes. After Mercuric chloride treatment, explants were thoroughly washed for 3-4 times with sterile water to remove any traces of Mercuric chloride.

**Culture media:**

The basal medium used for the culture is Murashige and Skoog medium (MS, 1962) with sucrose 3% , 0.8% agar and growth hormones.

**Table: 7 Preparation of Stock solution of MS medium**

| Constituents | Nutrient | Concentration stock (g/l) | Volume of stock per litre of medium (ml) |
|---|---|---|---|
| Macronutrients | $NH_4NO_3$ | 16.5 | 100 |
| | $KNO_3$ | 19 | |
| | $CaCl_2.2H2O$ | 4.4 | |
| | $MgSO_4.7H_2O$ | 3.7 | |
| | $KH_2PO_4$ | 1.7 | |
| Micronutrients | KI | 0.0415 | 10 |
| | $H_3BO_3$ | 0.31 | |
| | $MnSO_4.4H2O$ | 1.115 | |
| | $ZnSO_4.7H2O$ | 0.43 | |
| | $NaMoO_4.H2O$ | 0.0125 | |
| | $CuSO_4.5H20$ | 0.00125 | |
| | $CoCl_2.6H2O$ | 0.00125 | |
| Iron source | $FeSO_4.7H2O$ | 1.39 | 10 |
| | $Na_2EDTA.2H2O$ | 1.835 | |
| Vitamins | Nicotinic acid | 0.05 | 1 |
| | Pyrodixine-HCL | 0.05 | |
| | Thiamine-HCL | 0.01 | |
| | Glycine | 0.2 | |
| Other organics | Sucrose | 3000 | Added as solid |
| | Myo-inositol | 10 | |

**Preparation of MS medium (1 litre)** [140]

Macroelements (100ml) + Microelements (10ml) + Iron source (10ml)

↓

0.1 ml myo-inositol + 1ml vitamin

↓

20g sucrose

↓ Stir

Adjust to pH 5.7 – 5.8

↓

Add 8g agar

↓ Shake

Autoclaved at 121$^{o}$C for 15 minutes

↓

Let cooled in room temperature
Add plant growth regulator

↓

Autoclaved at 121$^{o}$C for 15 minutes
Pour into dish

**Preparation of PGR** [108-106]

Plant growth regulator (PGR) was prepared as 1mg/ml stock solution and stored in 4$^{o}$C prior to use. PGRs used for *D.sissoo* are BAP and NAA. The solvent for these PGR is 1 M NaOH. The effect of the PGR is to promote the formation of callus.

**Sterilization of media and PGR**

Medium preparation is autoclaved at 121$^{o}$C (250$^{o}$F) at 15 lb/in$_2$ (103.4kPa) for 15 minutes. PGR like NAA and BAP is not heat labile therefore, can be sterilized using autoclave machine.

Following are some of the supplements which were used in combination for the induction of callus, differentiation and multiple shoot formation.

i. Basal Medium (BM)

ii. BM+ BAP 0.5 mg /L + NAA 0.5 mg /L

iii. BM+ BAP 1mg /L + NAA 0.5 mg /L

iv. BM+ 1.5mg BAP/L + NAA 0.5 mg /L

v. BM+ BAP 2 mg /L + NAA 0.5 mg /L

vi. BM+ BAP 2.5mg /L + NAA 0.5 mg /L

vii. BM+ BAP 3mg /L + NAA 0.5 mg /L

viii. BM+ BAP 3.5mg /L + NAA 0.5 mg /L

ix. BM+ BAP 4mg /L + NAA 0.5 mg /L

The concentrated stock solutions of all the ingredients were prepared and stored under refrigeration. To prepare stock solution of micro salts, all the micro salts in required quantities were dissolved in one liter of distilled water and used as stock solution. Like wise stock solutions of all other ingredients are also prepared and kept under refrigeration. Similarly stock solutions of growth hormones were also prepared.

The medium was prepared by adding required quantities of all the ingredients in the conical flask. After adding all the ingredients in required amounts, the final volume is made up with the help of distilled water. pH of the medium is adjusted to 5.8 by using 1N KOH or 1N HCl. After adjusting the pH, agar is added to the medium at the rate of 0.8% w/v for

solidification of the medium. After pouring the media in bottles are tightly capped and labeled properly. After that media is autoclaved at $121^0$C for 20 minutes at 15psi.

**Inoculation of explants**

Under a laminar flow hood, after sterilization of explants, these were inoculated in culture bottles aseptically. For inoculation explants were transferred to large sterile glass petriplate or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed to make them in suitable sizes with sterile scalpel blade. After cutting explants into suitable size (2-3cm), explants are transferred to culture bottles containing medium. After inoculating the explants in culture bottle the mouth of bottle is quick flamed and bottles are tightly capped and mouths of the bottles were properly sealed to avoid entry of external air. After proper labelling clearly mentioning media code, date of inoculation etc. the bottles were transferred to growth room.

**Cultural conditions**

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± $4^o$C. The source of illumination consisted of 2.5 feet wide fluorescent tubes (40 watt) and incandescent bulb (25 watt). The intensity of illumination was 3500 lux at the level of cultures and a 12 hour light regime was followed by 12 hour darkness. The results were presented in table 8.

# CHAPTERVI

# PHYTO CHEMICAL STUDIES

Phytochemistry deals with natural product organic chemistry and plant biochemistry. It also deals with a variety of secondary metabolites that are produced by plants, their chemical structures, biosynthesis, metabolism, natural distribution and biological functions**.** For these operations, methods are needed for separation, purification and identification of the many different constituents present in plants [143].

The leaves of *Dalbergia sissoo* was collected in and around Madurai and authenticated by taxonomist.

The shadow dried leaves were powdered and then subjected to the following preliminary phytochemical studies.

## SECTION-A

## ORGANOLEPTIC EVALUATION

**Nature** - Coarse powder

**Colour** - Greenish yellow

**Odour** - Characteristic odour

**Taste** - Bitter followed by astringent taste

The powdered plant material and extracts were subjected to the following chemical tests and the results were tabulated in table 9.

## SECTION-B

## QUALITATIVE CHEMICAL EVALUATION

The chemical nature, specific identity, polarity, etc of the substances in the crude extract can be determined by a number of ways including wet chemical tests. In that a color reaction or precipitate is response to specific compound usually a class of compound. Such test can be useful for the investigation of the chemical compounds and to monitor the effectiveness of an extraction process. All the extracts were subjected to qualitative chemical analysis. The various tests performed on the extracts were for steroids, terpenoids, flavones, anthraquinones, sugars, glycosides, alkaloids, quinones, phenols and tannins and the results were tabulated in table 10.

### 1. TEST FOR STEROLS

The powdered leaf was first extracted with petroleum ether and evaporated to a residue. Then the residue was dissolved in chloroform and tested for sterols.

### a. SALKOWSKI'S TEST

A few drops of concentrated sulphuric acid was added to the above solution, shaken well and set aside. The lower chloroform layer of the solution turned red in color indicating the presence of sterols.

### b. LIEBERMANN – BURCHARD'S TEST

To the chloroform solution a few drops of acetic anhydride and 1 ml of concentrated sulphuric acid were added through the sides of the test tube and set aside for a while. At the junction of two layers a brown ring was formed. The upper layer turned green indicating the presence of sterols.

**2. TEST FOR CARBOHYDRATES**

**a. MOLISCH'S TEST**

The aqueous extract of the powdered leaf was treated with 2-3 drops of 1% alcoholic α-naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube.

A purple color indicates the presence of carbohydrates.

**b. FEHLING'S TEST**

The aqueous extract of the powdered leaf was treated with Fehling's solution I and II and heated on a boiling water bath for half an hour.

A red precipitate was obtained indicating the presence of free reducing sugars.

**c. BENEDICT'S TEST:**

The aqueous extract of the powdered leaf was treated with equal volume of Benedict's reagent.

A red precipitate was formed indicating the presence of reducing sugar.

**3. TEST FOR PROTEINS**

**a. MILLON'S TEST**

A small quantity of acidulous – alcoholic extract of the powdered drug was heated with Millon's reagent.

A white precipitate turning red on heating indicates the presence of proteins.

**b. BIURET TEST**

To one portion of acidulous – alcoholic extract of the powdered drug one ml of 10% sodium hydroxide solution was added, followed by this one drop of dilute copper sulphate solution was added.

Violet color was obtained indicating the presence of proteins.

**4. TEST FOR ALKALOIDS**

a. About 2gm of the powdered material was mixed with 1gm of calcium hydroxide and 5ml of water into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water bath. To this 200ml of chloroform was added, mixed well and refluxed for half an hour on a water bath. Then it was filtered and the chloroform was evaporated. To this 5ml of dilute hydrochloric acid was added followed by 2ml of each of the following reagents.

**a) MAYER'S TEST:**

To small quantity of the extract add Mayer's reagent. No cream colour precipitate indicates the absence of alkaloids.

**b) DRAGEN DORFF'S TEST:**

To small quantity of the extract add Dragendorff's reagent. No Orange brown precipitate indicates the absence of alkaloids.

**c) WAGNER'S TEST:**

To small quantity of extracts add Wagner's reagent. No Reddish brown precipitate indicates the absence of alkaloids.

**d) HAGER'S TEST:**

To small quantity of extracts add Hager's reagent. No Yellow precipitate indicates the absence of alkaloids.

**e) TEST FOR PURINE GROUP (MUREXIDE TEST)**

The residue obtained after the evaporation of chloroform as described in (a) was treated with 1ml of hydrochloric acid in a porcelain dish and 0.1gm of Potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the

vapour of dilute ammonia solution. No purple color was obtained indicating the absence of purine group of alkaloids.

**5. TEST FOR GLYCOSIDES**

**a) BORNTRAGER'S TEST**

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The organic layer was separated to which ammonia solution was added slowly.

Ammoniacal layer showed the pink colour indicating the presence of anthraquinone glycosides.

**b) MODIFIED BORNTRAGER'S TEST**

About 0.1g of the powdered drug was boiled for 2minutes with dilute hydrochloric acid and few drops of ferric chloride solution, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract.

Pink color was observed in ammoniacal layer showing the presence of anthraquinone glycosides.

**c) TEST FOR CARDIAC GLYCOSIDES(FOR DEOXYSUGAR)**

**i) KELLER KILIANI TEST**

About 1g of the powdered leaf was boiled with 10ml of 70% alcohol for 2 minutes, cooled and filtered. To the filtrate 10ml of water and 5 drops of solution of leads subacetate were added and filtered, evaporated to dryness. The residue was dissolved in 3 ml of glacial acetic acid. To these 2 drops of ferric chloride solution was added. Then 3 ml of concentrated sulphuric acid was added to the sides of the test tube carefully and observed.

Reddish brown layer was observed indicating the presence of deoxysugars of cardiac glycoside

**d) TEST FOR CYANOGENETIC GLYCOSIDES**

Small quantity of the powder was placed in a stoppered conical flask with just sufficient water, to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place.

No brick red color was produced on the paper indicating the absence of cyanogenetic glycosides.

**6. TEST FOR SAPONINS**

About 0.5g of the powdered drug was boiled gently for 2 minutes with 20ml of water and filtered while hot and allowed to cool. 5 ml of the filtrate was then diluted with water and shaken vigorously. No frothing was produced indicating the absence of saponins.

**7. TEST FOR TANNINS**

A small quantity of the powdered drug was extracted with water. To the aqueous extract few drops of ferric chloride solution was added.

A bluish black color was produced indicating the presence of tannins.

**8. TEST FOR FLAVONOIDS**

**a. SHINODA'S TEST**

A little of the powdered drug was heated with alcohol and filtered. To the test solution magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for five minutes.

Red colour was obtained indicating the presence of flavonoids.

**b. ALKALI TEST**

To the small quantity of test solution 10% aqueous sodium hydroxide Solution was added. Yellow orange color was produced indicating the presence of flavonoids.

**c. ACID TEST**

To the small quantity of test solution, few drops of concentrated sulphuric acid were added. Yellow orange colour was obtained indicates the presence of flavonoids.

**9. TEST FOR TERPENOIDS**

A little of the powdered leaf was extracted with chloroform and filtered. The filtrate was warmed gently with tin and thionylchloride. Pink color solution appeared which indicated the presence of terpenoids.

**10. TEST FOR THE PRESENCE OF VOLATILE OIL**

Weighted quantity (250 gm) of fresh leaves were extracted and subjected to hydro distillation using volatile oil estimation apparatus.

Volatile oil was obtained indicating the presence of volatile oil.

**11. TEST FOR MUCILAGE**

Few ml of aqueous extract prepared from the powdered crude drug was treated with ruthenium red. Red colour reaction indicating the presence of mucilage.

## SECTION – C

## QUANTITATIVE ESTIMATION OF PHYTO-CONSTITUENTS

### PREPARATION OF EXTRACTS

### PREPARATION OF ETHANOLIC EXTRACT OF *Dalbergia sissoo* [143]

The shade dried and coarsely powdered leaves of *Dalbergia sissoo* was defatted with petroleum ether (60-80˚C) for three days by triple maceration. The defatted marc was extracted with 70% ethanol by triple maceration and filtered. The filtrate was concentrated under reduced pressure to obtain a solid residue which was dark green in colour.

### EXTRACTION OF SECONDARY METABOLITE FROM CALLUS

Fresh calli (10.3g) with 50 ml of ethanol were homogenized at room temperature. The samples were shaken for 72 hours, and then centrifuged for 15min at 10,000 rpm on a centrifuge and the supernatant was collected. The supernatant was concentrated to get the residue for phytoconstituent analysis.

### ESTIMATIONS:

### I) TOTAL PHENOL DETERMINATION [144-147]

Phenols comprise the largest group of plant secondary metabolites. They range from simple structures with only one benzene ring to larger molecules such as tannins, anthraquinones, flavonoids and coumarins. They are defined as compounds that have at least one hydroxyl group attached to a benzene ring. Phenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activity.

**PRINCIPLE**

The total phenolic content of the various concentrations of extract of *Dalbergia sissoo* was determined by Folin Ciocalteu reagent method. All the phenolic compounds are oxidized by the Folin-Ciocalteu Reagent. This reagent is reduced during oxidation of the phenolic substances, into a mixture of blue molybdenum and tungsten oxides.

The blue colour produced has a maximum absorption at about 750-760nm. The absorption is proportional to the quantity of oxidized phenolic compounds.

**INSTRUMENT**

Shimadzu UV Visible spectrophotometer, Model 1800

**REAGENTS REQUIRED**

a) **Folin Ciocalteu Reagent (1N)**

The Folin Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate. Commercially available Folin Ciocalteu Reagent (2N) was diluted with an equal volume of distilled water. The resultant solution was kept in a brown color bottle and stored in refrigerator at 4 ºc.

b) **Sodium carbonate solution (10%)**

Sodium carbonates 350gm was dissolved & makes the volume upto 1000ml & heat the solution at 70-80°c, then cool & filter with glass wool & keep it overnight.

c) **Standard gallic acid solution**.

**PROCEDURE**

To the 1ml (1mg/ml and 0.5mg/ml) of ethanolic extracts of *Dalbergia sissoo,* and ethanolic extracts of *Dalbergia sissoo* from callus and 0.5ml of Folin Ciocalteu reagent

(1N) was added and allowed to stand for 15min. Then 1ml of 10% sodium carbonate solution was added to the above solution. Finally the mixtures were made upto 10 ml with distilled water and allowed to stand for 30 minutes at room temperature and total phenols were determined by spectrophotometrically at 760nm.

The calibration curve was generated by preparing gallic acid at different concentration (2, 4, 6, 8, 10µg/ml). The reaction mixture without sample was used as blank. Total phenol content of various leaf extracts are expressed in terms of mg of gallic acid equivalent per gm of extract (mg GAE/g). The results were tabulated in table 12 and the calibration graph was presented at Fig 6.1

**II) TOTAL FLAVONOID DETERMINATION [148-150]**

The word 'flavonoid' is derived from the latin word flavus meaning yellow and many flavonoids are indeed yellow in color. It consists of a single benzene ring joined to a benzo-gamma-pyrone structure. They are able to complex metal ions, acts as antioxidants and bind to proteins such as enzymes and structural proteins. The different classes within the group are distinguished by additional oxygen containing hetrocyclic rings and hydroxyl groups. These include the catechins, leucoanthocyanidins, flavanones, flavones, anthocyanidins, flavonols, chalcones, aurones and isoflavones.

**PRINCIPLE**

The aluminum chloride colorimetric technique was used for estimation of total flavonoid estimation. Aluminum ions form stable complexes with $C_4$ keto group and either to $C_3$ or $C_5$ hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavanoids. These

complexes showed a strong absorption at 415nm which is used for the estimation of flavonoids.

**INSTRUMENT**

Shimadzu UV Visible spectrophotometer, Model 1800

**REAGENTS REQUIRED**

10% aluminum chloride

1M potassium acetate

**PROCEDURE**

1mL of ethanolic extracts of *Dalbergia sissoo*, and ethanolic extracts of *Dalbergia sissoo* from callus at concentrations 50µg/mL and 100µg/mL were taken and0.1mL of aluminum chloride solution, 0.1mL of potassium acetate solution and 2.8mL of ethanol were added and the final volume was then made up to 5mL with distilled water. After 20min the absorbance was measured at 415nm.

A calibration curve was constructed by plotting absorbance reading of quercetin at different concentrations. The sample without aluminium chloride was used as a blank. The total flavonoid content in the extract was expressed as milligrams of quercetin equivalent per gram of extract and the results were tabulated in table 13. (Fig.6.2)

**III) TOTAL TANNIN DETERMINATION [71, 72]**

**PRINCIPLE**

Total tannin content of extract was determined by Folin Denis reagent method. Tannin like compounds reduces phosphor tungstomolybdic acid in alkaline solution to produce a highly blue coloured solution and the intensity of which is proportional to the amount of tannins and estimated by spectrophotometer at 700 nm.

**INSTRUMENT**

Shimadzu UV Visible spectrophotometer, Model 1800

**REAGENTS REQUIRED**

a) Folin Denis Reagent ( sodium tungstate 100g and phospho molybdic acid 20gm were dissolved in distilled water 750ml along with phosphoric acid 50ml. The mixture was refluxed for 2 hours and volume was made upto 1 litre with distilled water )

b) Sodium carbonate solution (10%)

c) Standard tannic acid solution.

**PROCEDURE**

0.2ml of (1mg/ml) ethanolic extracts of *Dalbergia sissoo*, was made upto1ml with distilled water. Then add 0.5ml of Folin Denis reagent and allowed to stand for 15 mins, then 1ml of sodium carbonate solution was added to the mixture and it was made upto 10ml with distilled water. The mixture was allowed to stand for 30mins at room temperature and the tannin content was determined spectrophotometrically at 700nm.

The calibration curve was generated by preparing tannic acid at different concentration (4, 8, 12, 16, 20µg/ml). The reaction mixture without sample was used as blank. The total tannin content in the leaf extract was expressed as milligrams of tannic acid equivalent per gm of extract. The results were tabulated in table 14. (Fig 6.3)

## SECTION – D

## ISOLATION OF ACTIVE CONSTITUENT

### ISOLATION, PURIFICATION AND IDENTIFICATION OF A COMPOUND FROM THE LEAVES OF *Dalbergia sissoo*

The need of compound isolation includes: (1) the generation and supply of larger amounts of an already known compound so that more extensive biological testing such as pharmacology and toxicology can be performed on the material, (2) the purification of a small amount of material for initial biological and chemical characterization to be performed, or (3) to purify sufficient material in order to conduct complete structural studies and further biological activity characterization [143].

### PRINCIPLE

The principle involved in the column chromatography for the separation of the compounds in adsorption at the solid liquid interface. For successful separation of a mixture, the compounds must show different degrees of affinity for the solid support and the interactions between adsorbent and compound must be reversible. As the adsorbent is washed with fresh solvent, the various components will therefore move down the adsorbent than with high affinity for the adsorbent.

The column consists of narrow bore tubing packed with finely divided inert solid that holds the stationary phase on its surface. The mobile phase occupies the open spaces between the particles of the packing. The components distribute themselves between the mobile phase and stationary phase. The elution occurs by forcing the sample components through the column by continuously adding fresh mobile phase. The average rate at which a solute migrates depends on the fraction of time it spends in that phase. Ideally the

resulting differences in rates cause the components in a mixture to separate into bands along the length of the column

**MATERIALS**

**COLUMN CHROMATOGRAPHY OF ETHANOLIC EXTRACT OF *Dalbergia sissoo***

The ethanolic extract (20g) was a dark green residue was chromatographed over 600g of silica gel (120 meshes) using hexane, petroleum ether, chloroform, methanol and their mixtures in various proportions in the order of their increasing polarities. The column was packed by using the suspension of silica gel in hexane.

Each 100ml of the eluate was collected and concentrated. The obtained fractions were tested for the presence of various constituents and nature of the compound.

**PREPARTION OF THE COLUMN** [156-159]

A glass column (length 100cm; diameter 3cm) was packed with activated silica gel in a form of slurry with hexane. The column was packed to a height of 65 cm in order to establish a column volume of 300ml.

The column was developed according to the following lines. The column was built up by passing two column volumes of hexane before the residue was loaded. The solvent was kept 5cm above the bed and the residue was carefully loaded in the form hexane slurry. The column was then developed with a series of solvent starting with n-hexane, petroleum ether, chloroform and methanol in increasing polarity.

The mobile phase consisted of hexane (100 ml), hexane **:** petroleum ether (90:10, 80:20, 70:30, 60:40, 50:50, 40:50, 40:60, 30:70, 20:80, 10:90) and petroleum ether (100

ml),chloroform (100 ml), chloroform **:** methanol (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90) and methanol(100 ml).

The different ratios with succeeding solvents were fixed and shown in the table no. and fractions of 100 ml were collected up to chloroform–methanol system and thereafter, fractions in smaller volumes were collected, checked with TLC using flavonoid solvent system (chloroform **:** methanol 9:1) and accordingly pooled, concentrated and processed further.

**PREPARATION OF THIN LAYER CHROMATOGRAPHY PLATES**

About 30gm of silica gel G was weighed out, and it was shaken with (10 ml) of distilled water to form a homogenous suspension. This suspension was poured into a TLC applicator which was 0 .25 mm thickness.

The plates were kept in the hot air oven at $110^{o}$ C for 1 hour to activate the silica gel G. The plates were then stored in a dry atmosphere and used whenever required

By using the capillary tube the compound elutes were spotted on TLC plates 2 cm above the bottom and subjected to chromatogram with different solvent systems. The compounds moved according to their affinity towards different solvent systems.

The plates after development in each solvent system were observed under UV lamp at 254 and 365 nm. The different spots developed in each solvent system were identified by means of different spraying systems and the results were tabulated in table 18. (Fig 5.1 & 5.2)

**Table: 16 Data showing the column chromatography of ethanolic extract**

| Fractions | Elutes | Nature of the residue | No. of spots |
|---|---|---|---|
| 1-4 | Hexane 100 mL | No residue | - |
| 5-8 | Hexane: petroleum ether (90:10) | Green brown | 3 |
| 9-14 | Hexane: petroleum ether (80:20) | Green brown | 2 |
| 15-18 | Hexane: petroleum ether (70:30) | pale green | 2 |
| 19-22 | Hexane: petroleum ether (60:40) | Green brown | 3 |
| 23-28 | Hexane: petroleum ether (50:50) | Brownish green | 3 |
| 29-34 | Hexane: petroleum ether (40:60) | Orange residue | 2 |
| 35-39 | Hexane: petroleum ether (30:70) | Greenish orange | 2 |
| 40-43 | Hexane: petroleum ether (20:80) | Pale green | 2 |
| 44-47 | Hexane: petroleum ether (10:90) | Pale green | 3 |
| 48-52 | Petroleum ether (100mL) | Yellowish orange | 3 |
| 53-59 | Chloroform (100 mL) | Yellowish green | 3 |
| 60-71 | Chloroform: Methanol (90:10) | Yellowish green | 1 |
| 72-78 | Chloroform: Methanol (80:20) | Greenish brown | 3 |
| 79-81 | Chloroform: Methanol (70:30) | pale green residue | 2 |
| 82-90 | Chloroform: Methanol (50:50) | Orange residue | 3 |
| 91-96 | Chloroform: Methanol (40:60) | Greenish residue | 3 |
| 97-106 | Chloroform: Methanol (30:70) | Yellow | 2 |
| 106-109 | Chloroform: Methanol (20:80) | Yellow | 2 |
| 110-118 | Chloroform: Methanol (10:90) | Pale yellow | 2 |
| 119-124 | Methanol (100) | Pale yellow | 2 |

## IDENTIFICATION OF ISOLATED COMPOUND

The compound was isolated from the dried leaves of *Dalbergia sissoo*.

Appearance : Yellowish powder

Solubility : Soluble in methanol and chloroform

Melting point : 255$^{o}$C

**MELTING POINT** [160]

The melting point of a substance is the temperature at which the material changes from a solid to a liquid state. Pure crystalline substances have a clear, sharply defined melting point. During the melting process, all of the energy added to a substance is consumed as heat of fusion, and the temperature remains constant.

Melting point is used to verify the identity and purity of isolated compound. The technique uses fairly simple instrumentation and requires very little material to accurately record a melting point, so it is often one of the first techniques used for identification of a compound. However, the technique is destructive and the material used is not recovered after obtaining the melting point.

The melting point was determined using a melting point apparatus (Sigma melting point apparatus, India)

## SECTION - E

## STRUCTURAL ELUCIDATION AND CHARACTERIZATION OF ISOLATED COMPOUNDS [143, 156]

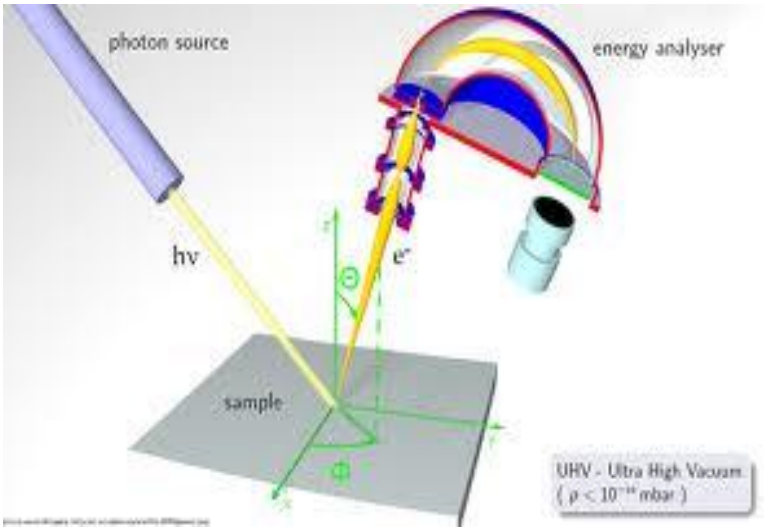
The different interactions of electromagnetic radiation with organic compounds based on their structural features forms the basis of the application of spectroscopy in the structural elucidation of organic compounds. The identification of compounds involves a diverse range of analytical techniques and methods such as nuclear magnetic resonance (NMR), ultraviolet (UV) and infrared (IR) spectroscopy, and mass spectroscopy (MS). In this study NMR and MS techniques were used as tools for the analysis and identification of the compounds isolated from *D.sissoo*. Structure elucidation of isolated compounds was achieved by a combination of nuclear magnetic resonance (NMR) and mass spectrometric (MS) analysis.

It was characterized by recording UV, FT-IR, MS and NMR spectra and melting point and comparing with the reported data (Enas Amin *et al.,* 2012).The melting point, Infra-red (IR), Mass, $^{1}$H-NMR spectra were recorded.

### ULTRA VIOLET SPECTROSCOPY:

An absorption in the UV-VIS region of the spectrum, i.e., the UV-VIS spectrum of a molecule indicates the presence of certain functional groups that have characteristic *n* – $\pi^*$, $\pi$- $\pi$ * transitions. These transitions are so characteristic that the absence of a UV-VIS spectrum for a molecule eliminates the presence of a number of functional groups in the molecule.

The λmax and the intensity of the absorption bands are indicative of the extent of conjugation in the molecule; larger the wavelength, greater the conjugation.



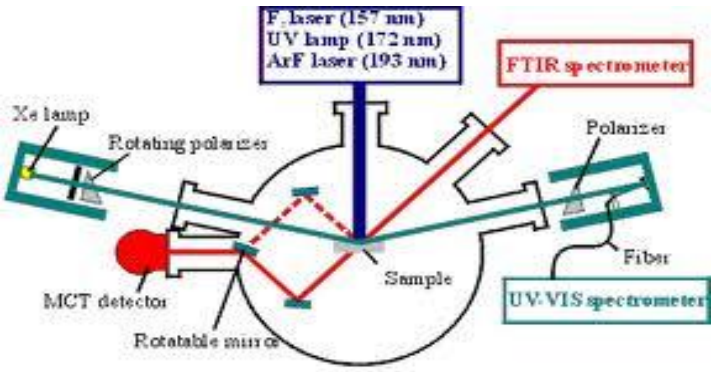

**Schematic representation of the UV spectrum**

The absorption maximum of isolated compound was identified using methanol and no bathochromic shift noted with $Alcl_3$ and bathochromic shift was present in potassium acetate. (Fig 5.3)

**FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR):**

An invaluable tool in organic structure determination and verification involves the class of electromagnetic (EM) radiation with frequencies between 4000 and 400 $cm^{-1}$ (wavenumbers). The category of EM radiation is termed infrared (IR) radiation, and its application to organic chemistry known as IR spectroscopy. Radiation in this region can be utilized in organic structure determination by making use of the fact that it is absorbed by interatomic bonds in organic compounds. Chemical bonds in different environments will absorb varying intensities and at varying frequencies. Thus IR spectroscopy involves collecting absorption information and analyzing it in the form of a spectrum. The frequencies at which there are absorptions of IR radiation ("peaks" or "signals") can be correlated directly to bonds within the compound.

Because each interatomic bond may vibrate in several different motions (stretching or bending), individual bonds may absorb at more than one IR frequency. Stretching absorptions usually produce stronger peaks than bending, however the weaker bending absorptions can be useful in differentiating similar types of bonds (e.g. aromatic substitution). It is also important to note that *symmetrical vibrations do not cause absorption of IR radiation.*




**Schematic representation of the FTIR**

IR spectrum was recorded using KBr disk method on IR spectrophotometer (Model 500, BUCK SCIENTIFIC) and results were tabulated in table 19. (Fig 5.4)

**NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)** [161]

NMR spectroscopy has been an important analytical tool for investigating natural compounds for many years. NMR analysis gives good quality information (e.g., composition, conformation) about the structures of simple natural compounds obtained from plants. The development of NMR allowed the reduction of the quantities required for structure elucidation and transformed completely this area of research. NMR spectroscopy can be used to identify the constitution and configuration of natural products, and to characterize their various physical and chemical properties. The NMR chemical shift is

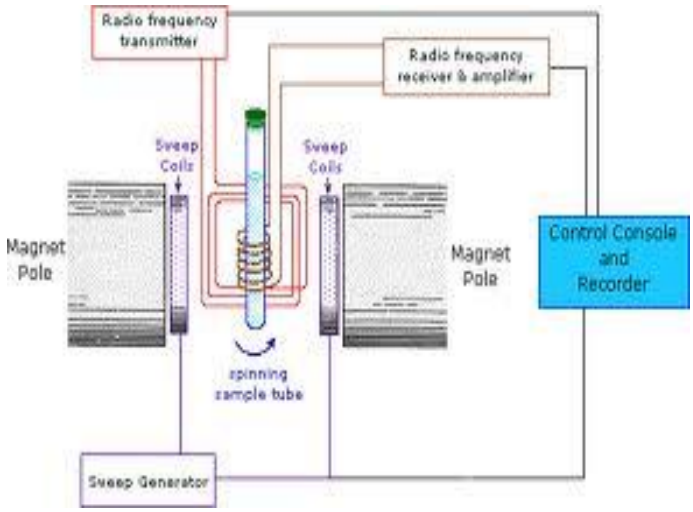
very sensitive to the electron distribution around the nucleus of an atom, is an important indicator of changes in the structural arrangement of molecules.

i) The number of different signals in the 1H-NMR spectrum indicates about the different types of protons present in the molecule.

ii) The position of the signals i.e. their chemical shift values, tells about the electronic environment of a particular proton.

iii) The area under the peaks obtained from the integrals for the signals of various types of protons provides information about the ratio of the numbers of different types of protons present in a molecule.

iv) The spin-spin splitting pattern of a particular signal gives information about the number of neighbouring protons present around the given type of protons.




**Schematic representation of the NMR**

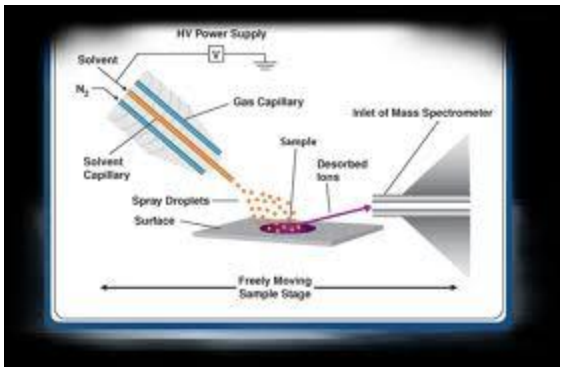
NMR was taken on av 300, $H^1$NMR (DMSO) 300 MHz, TMS as standard as shown in the table and the spectrum was attached. (Fig 5.5)

## MASS SPECTROSCOPY [145]

Mass Spectrometry is an important tool for the identification and structural elucidation of natural products. Mass Spectrometry (MS) was primarily used to obtain molecular weights.

Former ionization techniques, as electron ionization, limited this use to non polar, volatiles and thermostable substances. The improvement of softest ionization techniques allowed gradually the analysis of polar and thermolabile compounds, having currently unlimited physical or chemical properties to be analyzed by MS.

ESI: Electrospray ionization, process in which a liquid stream of dissolved analyte is broken up into small droplets by the action of a high potential.




**Schematic representation of the Mass spectra**

A mass spectrum was recorded in the positive ion mode on mass spectrophotometer (API 165, PERKINELMER). (Fig 5.6)

# CHAPTER-VII

# PHARMACOLOGICAL SCREENING

## SECTION- A

## IN VITRO ANTIOXIDANT ACTIVITY

Oxygen gives energy by oxidation of food. It is essential for living. During this process highly reactive and harmful oxygen species are also generated and it can damage living organisms. The free radicals initiate the oxidative stress and it is due to the imbalance between formation and neutralization of pro oxidants, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. Anti oxidants either prevent these reactive oxygen species or remove them before they can damage the cellular components. The anti oxidant effect is mainly due to the presence of phenolic compound such as flavonoids and phenolic diterpenes [162-163].

Hydroxyl radical scavenging activity, β-Carotene – linoleic acid assay, DPPH method, Superoxide radical scavenging activity, Nitric oxide radical inhibition assay, Reducing power method, Phosphomolybdenum method, Peroxy nitrile radical scavenging activity, Xanthine oxidase method, Ferric reducing ability of Plasma, Thiobarbituric acid assay etc are some of the methods.

### DETERMINATION OF SCAVENGING ACTIVITY AGAINST HYDROGEN PEROXIDE [164-165]

### PRINCIPLE

The radical scavenging activity against hydrogen peroxide of plant extract was determined by using the method of Janani *et al.* The principle is based on the capacity of

the extracts to decompose the hydrogen peroxide to water. $H_2O_2$ in the presence of **$O_2$ -** can generate highly reactive ˙OH hydroxyl radicals via the metal, the scavenging of $H_2O_2$ in cells is critical to avoid oxidative damage. Thus, the scavenging of hydrogen peroxide is an important antioxidant defence mechanism.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH + OH-$$

The decomposition of hydrogen peroxide to water involves the transfer of electrons as in Equation

$$H_2O_2 + 2H+ + 2e- \longrightarrow 2H2O$$

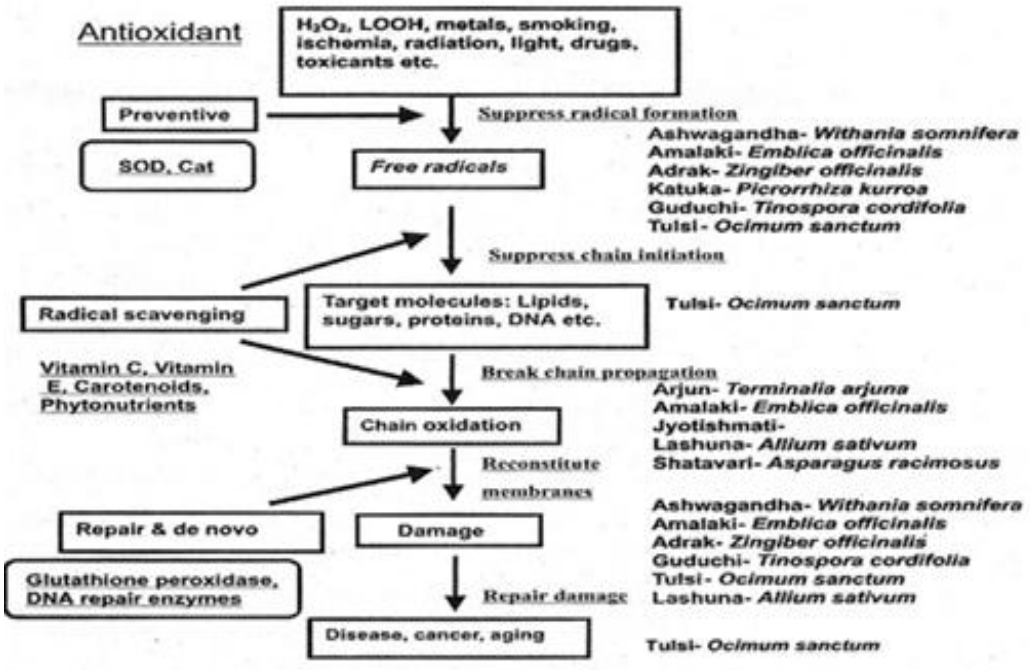



**Fig 7.1 Mechanism of anti oxidant**

**Reagents**

6% hydrogen peroxide diluted with water in the ratio of 1:10

0.1M, pH 7.4 phosphate buffer

**Procedure**

To the 1ml of test solutions at different concentrations were added to 3.8mL of 0.1M phosphate buffer solution (pH 7.4) and then mixed with 0.2mL of hydrogen peroxide solution. The absorbance of the reaction mixture was measured at 230nm after 10min. The reaction mixture without sample was used as blank. Sample blank was also prepared without reagents.Ascorbic acid was used as standard. The percentage inhibition of hydrogen peroxide was calculated using the formula

$$\% \text{ inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100$$

The concentration of the sample required for 50% reduction in absorbance ($IC_{50}$) was calculated using linear regression analysis. The results obtained are presented in table 22 and Fig. 7.2

**DIPHENYLPICRYLHYDRAZYL (DPPH) METHOD** [166-169]

**Principle**

DPPH is a stable free radical with a distinctive ESR signal. Its reaction with antioxidants can be followed by the loss of absorbance at 517nm. It is widely accepted that DPPH accept an electron or hydrogen radical and become a stable diamagnetic molecule. Due to its odd electron, the ethanol solution of DPPH (purple colour solution) shows a strong absorption at 517nm. DPPH radicals react with suitable reducing agents where the pairing of electrons takes place and the solution loses colour stochiometrically with the number of electrons taken up.

$$DPPH^{\cdot} + AH \rightarrow DPPH\text{-}H + A^{\cdot}$$

**Instrument**

Shimadzu UV Visible spectrophotometer, Model 1800

**Reagents**

0.1mM diphenylpicrylhydrazyl in ethanol

**Procedure**

A stock solution of DPPH was prepared in ethanol (4mg/100ml). To the 1mL of test samples of different concentrations 4mL of DPPH was added. Control without test compound was prepared in an identical manner. Blank was prepared in the similar way, where DPPH was replaced by ethanol. The reaction was allowed to be completed in the dark for about 30min. Then the absorbance of test mixtures was read at 517nm. The percentage inhibition was calculated and expressed as percent scavenging of DPPH radical. Vitamin C was used as standard.

The percentage scavenging was calculated using the formula

$$\% \text{ inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100$$

The concentration of the sample required for 50% reduction in absorbance ($IC_{50}$) was calculated using linear regression analysis. The results obtained are presented in Table 23 and Fig 7.3

**TOTAL ANTIOXIDANT ACTIVITY BY PHOSPHOMOLYBDENUM METHOD** [170-172]

**Principle**

The total antioxidant activity of the extract was evaluated by phosphomolybdenum method. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample and by the subsequent formation of green phosphate Mo (V) complex at acidic pH which has a

maximum absorption at 695nm. This method is routinely used to determine total antioxidant activity of samples.

$$Mo^{6+} \rightarrow Mo^{5+}$$

**Instrument**

Shimadzu UV Visible spectrophotometer, Model 1800

**Reagents**

0.6M sulphuric acid

28mM sodium phosphate

4mM ammonium molybdate

**Procedure**

An aliquot of 0.3ml of different concentrations of sample solutions was combined with 2.7ml of the reagent solution ($H_2SO_4$, sodium phosphate and ammonium molybdate). In case of blank, 0.3mL of methanol was used in place of sample. The tubes were incubated in a boiling water bath at 95°C for 90min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695nm against blank. The standard vitamin C was treated in a similar manner. The antioxidant activity was expressed as equivalents of Vitamin C (µg/g). The results are tabulated in table 24 and Fig 7.4.

## SECTION- B

## PANCREATIC LIPSAE INHIBITON ASSAY

### PANCREATIC LIPASE

#### A) Principle

Pancreatic lipase is also known as pancreatic triacylglycerol lipase. It is secreted from the pancreas, and it hydrolyses (breaks down) dietary fat molecules in the human digestive system, converting triglyceride into monoglycerides and free fatty acids [173, 174].

Triacylglycerol + $H_2O$ diacylglycerol + a fatty acid anion

Bile salts secreted from the liver and stored in gallbladder are released into the duodenum and emulsify large fat droplets into smaller droplets, thus increasing the surface area of the fat, which allows the lipase to break apart the fat more effectively. The resulting monomers (2 free fatty acids and one 2-monoacylglycerol) are then moved by way of peristalsis along the small intestine to be absorbed into the lymphatic system by a specialized vessel called a lacteal. (Fig 8.1)

An anti obesity drug – Orlistat, which inhibit pancreatic lipases in the lumen of the gastrointestinal tract to decrease systemic absorption of dietary fat.

#### B) Materials and methods

- Chicken (*Gallus domesticus*) pancreas
- Sucrose solution (0.01M)
- Ammonium sulphate (50% saturation)
- Phosphate buffer (pH 7)
- Olive oil

- ❖ Orlistat (60mg)
- ❖ Pancreatic lipase and extract- Ethanolic extract of leaves of *Dalbergia sissoo* & dalpatein

**C) Extraction of lipase from chicken (*Gallus domesticus*) pancreas** [175-183]

Pancreas of freshly slaughtered chicken was collected with the guidance of vertinary surgeon. It was washed and pancreatic lipase placed in ice cold sucrose solution (0.01M). The pancreas was homogenized in 0.01M sucrose and centrifuged. The supernatant was separated and subjected to ammonium sulphate precipitation (50% saturation). The obtained pellet after centrifugation was dissolved in sucrose solution and again saturated with 50% ammonium sulphate and centrifuged. Finally pellets were used as enzyme source by dissolving in Phosphate buffer (pH 7).

**D) Determination of chicken pancreatic lipase activity**

The chicken pancreatic Lipase activity was determined by incubating an emulsion containing 8ml of Olive oil (Dietary fat), 0.4ml of phosphate buffer and 1ml of chicken pancreatic lipase for an hour, the reaction was stopped by addition of 1.5 ml of a mixture solution containing acetone and 95% ethanol (1:1). Appearance of pink color from yellow color shows the liberated fatty acids, which was determined by titrating the solution against 0.02M NaOH (standardized by potassium hydrogen phthalate) using phenolphthalein as an indicator.

**E) Pancreatic lipase inhibitory activity**

Pancreatic lipase inhibitory activity was studied using Anilkumar *et al.,* method with slight modification. Standard drug and test compounds were prepared in different concentrations such as 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, 2.5 mg/mL, and

3.0 mg/mL. A 100µl of each concentration of sample pancreatic lipase was mixed with 8ml of olive oil, 0.4ml of phosphate buffer and 1ml of chicken pancreatic lipase and it was incubated for 60 minutes. The reaction was stopped by adding 1.5 ml of a mixture solution containing acetone and 95% ethanol (1:1). The liberated fatty acids were determined by titrating the solution against 0.02M NaOH using phenolphthalein as indicator and the percentage inhibition of lipase activity was calculated using the formula:

$$\text{Lipase inhibition} = A - B / A \times 100$$

Where A is lipase activity, B is activity of lipase when incubated with the standard and test compounds and the results were tabulated in table 25. (Fig 8.2)

## SECTION- C

## EVALUATION OF THE TOXIC EFFECTS OF *Dalbergia sissoo* IN ZEBRA FISH (*Danio rerio*) EMBRYOS

### Introduction

**The emergence of zebrafish in the scientific world** [204-209]

During the past few decades, zebrafish has emerged as one of the most important and popular experimental animals for the scientific community. According to the zebrafish information network (ZFIN), there are more than 500 world-wide research groups that use this animal as a model system to address various research topics ranging from basic to applied science.

Zebrafish embryo toxicology studies include

- Toxic effects of drugs in Zebrafish embryos
- Acute toxicology model system
- Teratogenicity, organ-specific toxicity
- High-throughput cellular cytotoxicity screening

Acute toxicity studies in Zebrafish (*Danio rerio*) may use to determine the toxicity dose of the drug and also fix the effective dose of the drug.

**Zebrafish as a model vertebrate**

There are several important features of zebrafish to make it an ideal experimental animal.

- ❖ Differences in the appearance between male and female zebrafish are easily distinguishable.
- ❖ In the laboratory, a pair of zebrafish can produce approximately 30-50 embryos per spawning,

- Zebrafish eggs are transparent and relatively large (~0.7 mm in diameter)
- Embryogenesis is rapid and all major organs develop within 24 hr.
- The generation time is also relatively short requiring 3-4 months.
- Therefore, it was possible to use zebrafish for the first vertebrate large-scale genetic screens which generated a large number of mutants with various phenotypes.
- A comprehensive range of powerful tools and research resources specific to zebrafish have been established.

Zebrafish offers **an alternative model system to assess the cytotoxic effects** of pre-clinical compounds on the developing embyro. Zebrafish share 80% homology with humans. The real benefit of this system is the ability to monitor cytotoxic effects in high-throughput multi-well assay plates.

Specialty assays include acute toxicity, teratogenecity, **organ-specific toxicity,** toxicogenomics, ecotoxicology, and nanotoxcity. Zebrafish embryo toxicity studies offer a well-established paradigm for testing potential therapeutics and required assays for IND (Investigational New Drugs) submission of new pre-clinical drugs.

**ACUTE TOXICITY STUDIES OF ZEBRAFISH EMBRYOS**

**AIM OF THE STUDY**

This study is designed to evaluate the acute toxicity of ethanolic extract and dalpatein from *Dalbergia sissoo* on newly fertilized zebrafish eggs for up to 72 hrs and is expected to reflect acute toxicity in fish in general. After 24, 48 and 72 hrs, five apical endpoints are recorded as indicators of acute lethality in fish which are as follows;

(i) Coagulation of fertilized eggs,

(ii) Lack of somite formation,

(iii) Lack of detachment of the tail-bud from the yolk sac,

(iv) Lack of heart-beat.

(v) Death

**PRINCIPLE OF THE TEST** [209]

Zebrafish embryos are individually exposed in 24-well microtiter plates to a range of concentrations of the test substance. The test is initiated immediately after fertilization and is continued for 72 hours. Lethal effects, as described by five apical endpoints, are determined by comparison with controls to identify the $LC_{50}$ values. The test method is based on using a minimum of five test concentrations as well as appropriate controls, with 5-10 individual embryos per exposure concentration. Each substance should be tested in parallel in two to three independent replicates. (Fig 9.1 & 9.2)

**MATERIALS AND METHODS**

**EXPERIMENTAL ANIMALS**

Adult zebra fish (*Danio rerio*) were procured from SNP aquarium in Nagercoil, Tamil Nadu, India and were allowed to adapt to laboratory conditions for 4 weeks before the commencement of the project.

**Maintenance of animal:**

The animals were kept in aquaria within a laboratory at 26 ± $1^0$C and a 12 – h light /dark cycle was maintained. Standardized water (ISO 7346 – 1, 1996) was used for the maintenance of the adult zebra fish. It was prepared from deionised water and the following salts were added: (Hank's solution) $CaCl_2$ x $2H_2O$ (117.6 mg/L), $MgSO_4$ x $7H_2O$ (49.3mg/L), $NaHCO_3$ (25.9mg/L) and KCl (2.3 mg/L) to produce standardized water. The

adult zebra fish were fed with newly hatched *Artemia nauplii* 2 times daily. The animals were cared in accordance with the guidelines to carry the experimental animals [210].

**Table: 26 Maintenance, breeding and typical conditions for embryo toxicity tests with the common OECD test fish species**

| **Parameters** | **Zebrafish (*Danio rerio*)** |
|---|---|
| Male to female ratio for breeding | 2:1 |
| Breeding tanks | 4 L tanks equipped with steel grid bottom and plant dummy as spawning stimulant |
| Egg structure and appearance | Stable chorion, highly transparent, non-sticky, diameter: ~0.7 mm |
| Embryo development at 26 °C 18 h: | Development of somites<br>21 h: Tail detachment<br>26 h: Heart-beat visible<br>28 h: Blood circulation<br>72 h: Hatching |
| Test type | Static or semi-static, 26 °C, 24-well plates (2 ml per cavity) |
| Major toxicological endpoints at 25°C | 24 h: Coagulation, tail detachment, somite development<br>48 h: Heart-beat visible |

**The Fish Embryo toxicity Test (FET)**

**Egg production**

The eggs were obtained from adult male and female zebra fish placed in breeding tanks for spawning in a ratio of 2:1. The funnels were filled with 10 –12 litres of standardized water. The eggs were obtained from the breeding funnels the following day between 30 and 60 minutes after light was switched on in the laboratory. Mating and

spawning take place within 30 minutes after turning on the lights. To prevent adult zebrafish from egg predation, the egg traps are covered with stainless steel mesh with a grid size of 2 mm. Plant imitations of plastic or glass is serving as spawning substrate are fastened to the mesh. About 20-30 minutes after the onset of light, the egg traps are removed and the eggs are collected. The traps should be replaced into the spawning tanks at the latest possible time or on the next day before the light is turned on. The collection of the fish eggs may take place in the maintenance tanks or in separated spawning tanks. A single mature female spawns about 100 - 200 eggs per day. The fertilization rate should be ≥50%. In case of fish spawning for the first time, fertilization rates may be lower in the first few spawns. The brood fish were thereafter returned to the aquaria.

**Test solutions** [211-212]

Test solutions of the selected concentrations are prepared by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test substance in the dilution water by mechanical means (*e.g.,* stirring or ultrasonification). Dimethylsulfoxide (DMSO) proved to be a useful solubilizing agent; acetone should be avoided, since it has been shown to produce side effects.

Stock solutions of Dalpatein and DSEE were prepared in dimethylsulphoxide (>99.5% DMSO) and then diluted 1:1000 in standardized water to exposure concentrations of 1 µg/L, 10 µg/L, 100 µg/L, 1mg/L and 10 mg/L. Standardized water and DMSO were used as negative controls. Newly laid eggs were placed in petri dishes containing the test solutions with the aid of a pipette.

**Start of exposure and duration of test:**

The eggs were examined in a microscope for the selection of eggs that had reached the five – cell stage. For each exposure concentration, 10 eggs were selected and transferred individually to 24 micro – well plates (Costar, Corning Incorporated, USA) with 250 μL of the test solutions. The plates were covered with parafilm and then kept at $26^0$C in a 12 – h light/dark cycle. The examination of the eggs was carried out with the aid of a microscope at 24, 48 and 72 hours post fertilization (hpf). Photos of the micro-well plates were taken with a mounted digital camera (Canon Power Shot ProI) and the results were tabulated in table 27. (Fig 9.5, 9.6, 9.7, 9.8 & 9.9)

**Probit analysis** [244-246]

According to probit analysis the $LC_{50}$ values of DSEE and Dalpatein were calculated. The percentage lethality or probit value versus log concentration was plotted and Y=50 is substituted in the resulting linear equation to obtain the X value. The antilog of X was then the $LC_{50}$ (conc. of 50 lethality) value. From the table it can be seen that the extract and Dalpatein possessed very low toxicity to zebrafish embryo with $LC_{50}$ of 10mg and 100 mg respectively which ensures the biosafety of the extract.

The corrected(%) mortality was calculated by using Schneider-Orelli's formula.

Schneider-Orelli's formula.

$$\text{Corrected \%} = \frac{\text{(Mortality \% in treated plot - Mortality \% in control plot)}}{\text{100 - Mortality \% in control plot}} * 100$$

## SECTION-D

## ANTI OBESITY AND LIPID LOWERING EFFECT OF *Dalbergia sissoo* USING ZEBRAFISH (*Danio rerio*)

### Introduction

Drug discovery involves a complex process of biochemical and cellular assays, with final validation in animal models, and ultimately in humans. Mammalian models of absorption, distribution, metabolism and excretion (ADME)/pharmacokinetics and efficacy are expensive, laborious and consume large quantities of precious compounds. There is also increasing pressure to limit animal use to situations in which they are absolutely necessary, such as in preclinical toxicity and safety assessment. Zebrafish are beginning to be used at various stages of the drug discovery process and can be a useful and cost-effective alternative when compared to some mammalian models (such as rodents, dogs and pigs).

The zebrafish (*Danio rerio*) has proven an excellent model for study of vertebrate development and genetics. We believe that zebrafish will prove a valuable model for study of human diseases, and we have sought to characterize some of the basic features of mature zebrafish. Accordingly, blood was collected from adult zebrafish and was analyzed for biochemical parameters. In our study, we use zebrafish in target validation, disease modeling, target and lead compound discovery and toxicology [233].

As a vertebrate, zebrafish possess many structural similarities with humans that worms and flies do not and have been used animal model for various human diseases [234-236].For example, zebrafish digestive organs, adipose tissues and skeletal muscle are

physically arranged similar to the human counterparts. Neural and endocrine signals regulating food intake are also conserved in zebrafish, including agouti-related protein (AgRP), leptin and adiponectin. The zebrafish larvae have been used in genetic and chemical screening experiments to identify novel genes involved in the regulation of energy homeostasis and potential therapeutic targets to treat obesity.

Zebrafish possess many of the same digestive organs, cells, and genes similar to humans and are currently being used as models of metabolic disorders such as diet-induced obesity, atherosclerosis, and diabetes. At the onset of exogenous feeding at approximately 5 days post fertilization (dpf), zebrafish larvae possess a functional digestive system and remain largely transparent, allowing them to be utilized for studies of digestive physiology in vivo (Farber *et al.*, 2001).

**Principle** [235-238]

Like the mammalian system, adult fish adipocytes store neutral lipid in the form of triacylglycerol and these stores are mobilized during periods of nutrient deprivation. Fat deposition in fish is also sensitive to nutrient availability and the enzymatic machinery responsible for fat deposition and mobilization in fish appears to be similar to that found in mammals. Cultured fish adipocytes express genes that are homologous to mammalian adipocyte markers [e.g., *Peroxisome proliferator- activated receptor* γ (*Pparg*), *CCAAT/enhancer binding protein*, *Lipoprotein lipase*, and *Leptin*] and are also responsive to many of the same endocrine signals as mammalian adipocytes.

The dietary fat consumed by humans and zebrafish are alike and consists primarily of triacylglycerol (TAG), which must be processed by digestive enzymes and bile for intestinal absorption. The pancreatic acinar cells produce, package, and secrete digestive

enzymes into the pancreatic ducts by fusion of zymogen granules with the api calacinar cell membrane.

After draining into the common bile duct, pancreatic lipases mix with bile derived from the liver. Hepatocytes drain bile into 'little canals' termed canaliculi, which direct bile into hepatic ducts which empty into the gall bladder. In response to stimulation by the hormone cholecystokinin (CCK), bile and fat-splitting enzymes in the gall bladder are pumped into the intestinal lumen to emulsify TAG by forming mixed micelles.

**Aim of the study**

To investigate the anti obesity and lipid lowering effect of *Dalbergia sissoo* Roxb in vertebrate animals, we used a zebrafish (*Danio rerio*) model, in which hypercholesterolemia is induced by high cholesterol (HC) diet, to mimic early obesity and its complications.

**Materials and methods**

**Materials**

Ethanolic extract of *Dalbergia sissoo* leaves (DSEE)

Dalpatein

Orlistat 60 mg (Standard marketed anti obesity drug)

**Chemicals**

Taiyo fish feed and Tubifex worms (High fat feed) supplied by TAIYO Pet products Pvt, Ltd, Chennai. All other chemicals used for this study were of analytical grade.

**Experimental animals**

Adult zebra fish were used for this study. They were housed under standard laboratory environment.

**Experimental protocol** [218]

Adult zebra fish (*Danio rerio*) were procured from SNP aquarium in Nagercoil, Tamilnadu, India and were allowed to adapt to laboratory conditions for 1 week before the commencement of the experiment as per ethical principles **(SBCP/2011-2012/IAEC/CPCSEA/1)**.

Fish were housed in groups of10 animals/tank were kept in aquaria within the laboratory at 26 ± $1^0$C and a 12 – h light /dark cycle was maintained. Standardized water (ISO 7346 – 1, 1996) was used for the maintenance of the adult zebra fish. It was prepared from deionised water and the following salts were added: $CaCl_2$ x $2H_2O$ (117.6 mg/L), $MgSO_4$ x $7H_2O$ (49.3mg/L), $NaHCO_3$ (25.9mg/L) and KCl (2.3 mg/L) (Sigma Aldrich) to produce standardized water.

Seven groups, each comprising ten zebrafish (*Danio rerio*) were employed in this study.

| **Animals n=10** | **Treatment** |
|---|---|
| Group I | Normal diet group |
| Group II | High cholesterol diet group(Control) |
| Group III | Treated with high cholesterol diet containing 50µg orlistat (Standard drug) |
| Group IV | Treated with high cholesterol diet containing 5% *Dalbergia sissoo* ethanolic extract (DSEE) |
| Group V | Treated with high cholesterol diet containing 10% *Dalbergia sissoo* ethanolic extract (DSEE) |
| Group VI | Treated with high cholesterol diet containing 25µg Dalpatein |
| Group VII | Treated with high cholesterol diet containing 50µg Dalpatein |

Each group (n = 10) consumed the assigned diet (5mg/ 10 mg/day/fish). A high cholesterol (HC) diet (Tubifex worms – containing 52% crude protein, 8% crude fat,7% crude fibre, 8%moisture). The high cholesterol feed is given to the animals except group I. The powder of crude extract and dalpatein were mixed with feed.

***GROUP I [Normal group]***

Normal feed Diet = 10 mg/day/fish

(Normal feed without 4% cholesterol)

(n=10) = 100 mg/day as BID for 4 weeks

***Group II [Control group]***

High Cholesterol (HC) Diet = 10 mg/day/fish

(Tube worm with 4 % cholesterol)

(n=10) = 100 mg/day as BID for 4 weeks

***Group III [Orlistat group]***

HC diet + 50 µg Orlistat

(HC diet 10 mg/day/fish + Orlistat (50µg) /day/ fish)

(n=10) = 100.5 mg /day as BID for 4 weeks

***Group IV [DSEE group- 5%]***

HC diet + DSEE (5%)

(HC diet 10 mg/day/fish + Crude extract (DSEE) 1 mg/day/ fish)

(n=10) = 100.5 mg/ day as BID for 4 weeks

***Group V [DSEE group- 10%]***

HC diet + DSEE (10%)

(HC diet 10 mg/day/fish + Crude extract (DSEE) 1 mg/day/ fish)

(n=10) = 110 mg/ day as BID for 4 weeks

***Group VI [Dalpatein- 25 µg]***

HC diet + Dalpatein

(HC diet 10 mg/day/fish + Dalpatein 25µg/day/ fish)

(n=10) = 100.25 mg/ day as BID for 4 weeks

***Group VII [Dalpatein- 50µg]***

HC diet + Dalpatein50µg

(HC diet 10 mg/day/fish + Dalpatein50µg/day/ fish)

(n=10) = 100.5 mg/ day as BID for 4 weeks

**Blood collection and biochemical estimation** [218]

After feeding for 4 weeks of feeding, 2 µL of blood was drawn from the heart of adult fish and combined with 5 µL of PBS, then collected into vaccutainer. Then it was centrifuged for 10 min at 2,500 rpm to get serum; then it was pipetted off the top. Serum from zebrafish were then subjected to assay biochemical parameters like serum total cholesterol (TC), HDL-cholesterol, and triglyceride (TG), Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) using a commercial semi auto analyser.

**Statistical analysis**

In biological experiments, the data (results) obtained at the end of the experiment is put to statistical analysis to determine whether the effect produced by a compound under study is genuine and not due to chance. Hence, the analysis usually attaches a test of statistical significance. The first step in such a test is to state the *null hypothesis*. When the *null hypothesis* is accepted, the difference between the two groups is not significant. In other words, both samples were indeed drawn from a single population, and the difference observed between the two groups was due to chance. If the *null hypothesis* is rejected, then the difference is significant. A difference between the treated and the control group which would have arisen by chance in less than 5% of cases, is considered as statistically

significant ($P<0.05$); that arising in less than 1% of cases as highly significant($P<0.01$); while that arising in less than 0.1% of cases as very highly significant ($P<0.001$).

**Arithmetic mean (X):** The mean of a number of individual values (X) is always nearer the true value than the individual value itself. $X = \epsilon X/n$.

**Standard deviation (SD):** In addition to the mean, the degree of variability of the responses has to be indicated since the same mean may be obtained from different sets of values. Standard deviation describes the variability of observations about the mean and is calculated using $\sqrt{\epsilon(X-X)^2/n-1}$.

# CHAPTER VIII

## COMPUTATIONAL STUDIES FOR ANTIOBESITY AND LIPID LOWERING EFFECTS OF *Dalbergia sissoo* Roxb.

**Docking –** Computational simulation of a candidate ligand binding to a receptor.

In the field of molecular modeling, **docking** is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules using for example scoring functions. The associations between biologically relevant molecules such as proteins, nucleic acids, carbohydrates, and lipids play a central role in signal transduction. Furthermore, the relative orientation of the two interacting partners may affect the type of signal produced (e.g., agonism vs. antagonism). Therefore docking is useful for predicting both the strength and type of signal produced.

Docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to in turn predict the affinity and activity of the small molecule. Hence docking plays an important role in the rational design of drugs. Given the biological and pharmaceutical significance of molecular docking, considerable efforts have been directed towards improving the methods used to predict docking.

### AIM OF THE STUDY

Physiologically, obesity is a disarray of energy balance and primarily considered as a disorder of lipid metabolism. A growing number of enzymes involved in lipid metabolic pathways are being identified and characterized. They represent a rich pool of potential

therapeutic targets for obesity. Inhibition of PL (triacylgycerol acyl hydrolase), the principal lipolytic enzyme, synthesized and secreted by pancreas is one of the approaches for the development of newer antiobesity drugs. Tetrahydrolipstatin (Orlistat), a commercial anti-obesity drug, is a known pancreatic lipase inhibitor. Recently, much interest has been shifted on plant flavonoids that might be beneficial in reducing the risk of obesity. Accordingly, investigation on the metabolic effects of plant flavonoids might lead to more effective strategies for the treatment of obesity. The health hazards like diabetes, obesity and metabolic related disorders are related to the dietary habits and most of the nutraceuticals on the market focuses on these areas.

In the present work, an integrative in vivo-in vitro-in silico approach was followed to study the antiobesity properties of isoflavonoid Dalpatein isolated from leaves of *Dalbergia sissoo* for their pancreatic Lipase (PL) inhibitory action. In order to understand the mode of binding of these compounds with the active site of the PL enzyme, molecular docking approach was employed.

**7-hydroxy-6-methoxy-3-(6-methoxy-1, 3-benzodioxol-5-yl)-4*H*-chromen-4-one(Dalpatein) to PL-Colipase complex (1LPB)**

Docking calculations were carried out using DockingServer[248]. The MMFF94 force field (*Halgren, 1998*) was used for energy minimization of ligand molecule (***dalpatein***) using DockingServer. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on ***PL-Colipase complex*** protein model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools. Affinity (grid) maps of 20×20×20 Å grid points and 0.375 Å spacing were generated using the Autogrid program [249 & 250]. AutoDock parameter set-

and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively.

Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method[251]. Initial position, orientation, and torsions of the ligand molecules were set randomly.

**Docking Parameters for Protein Clean**

| | |
|---|---|
| ***Tstep*** | **0.2** |
| ***Qstep*** | **5.0** |
| ***Dstep*** | **5.0** |
| ***Rmstol*** | **2.0** |
| ***ga_pop_size*** | **150** |
| ***ga_num_evals*** | **250000** |
| ***ga_num_generations*** | **540000** |
| ***ga_run*** | **2** |

Each docking experiment was derived from 10 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.

# CHAPTER IX

# RESULT AND DISCUSSION

*Dalbergia sissoo* is also called Indian rosewood, belongs to the family Fabaceae. It is a large deciduous perennial tree found throughout India. Various phyto constituents has been reported in this plant such as flavanoids, tannins, glycosides and phenols. This plant has been reported to possess antidiabetic, antipyretic, aphrodisiac, expectorant, antibacterial and antifungal activities. *Dalbergia sissoo* oil has reported for repellant activity against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*, and also resistant to some wood boring insects. The roots of *D. sissoo* are reported in the treatment of uterine muscle contracting activity and the leaves are reported for the treatment of gonorrhea, leucorrhea and wound healing.

This dissertation work includes the pharmacognostical, callus induction, phytochemical, pharmacological and in silico studies on the leaves of *Dalbergia sissoo*.

**Chapter 1**

Introduction of this dissertation work has been discussed in this chapter which gives information about obesity and human health, Causes and medical complications of obesity, drug molecules for obesity, natural products in drug discovery and modern medicine, approaches towards evaluation of medicinal plants prior to clinical trials, safety and pharmacovigilance, integration of *in silico* screening and natural products were discussed in this chapter.

**Chapter 2**

Literature survey pertaining to the pharmacognostical, phytochemical, pharmacological and molecular level studies of *Dalbergia sissoo.* The ethnomedical

information revealed that the plant is used for the treatment of eye ailments, abortifacient, anthelmintic, antipyretic, aphrodisiac, expectorant, refrigerant, anal disorders, blood diseases, burning sensations, dysentery, dyspepsia, leucoderma and skin ailments. It is reported for the activities for anti-inflammatory, anti diabetic, larvicidal, antioxidant, antibacterial, antiprotozoal, and antiviral activities.

**Chapter 3**

Our aim is to standardize this drug by means of various pharmacognostic parameters, isolation and characterization of active principle, evaluation of both extracts and active principle for pharmacological screening to prove the ethnomedical claim of anti obesity

**PHARMACOGNOSTICAL STUDIES**

Pharmacognostical works are ensuring the quality, purity of starting material by creating numerical values of standards for comparison. To ensure reproducible quality of herbal plant, authentication is essential. Pharmacognostical parameters for easy identification like leaf constants, microscopy & physico chemical analyses are few of the basic standardization of herbals. According to WHO the macroscopical and microscopical description of a medicinal plant is the first step towards the establishment of identity and the degree of purity of such material. The pharmacognostical parameters are major reliable and inexpensive criteria for conformation of the crude drugs. Hence, in this dissertation the pharmacognostical standardization has been performed for the leaf of the plant.

**Macroscopical studies**

The leaves are alternately arranged oddly pinnate, bifarious and tapering to the point with cuneate base. Inflorescences are axillary panicle, composed of several short spikes with sessile to subsessile flowers. Bract is small, pubescent and caducous. Calyx is 5 mm long,

teeth ciliate, and unequal shorter than the tube. Corolla is yellowish white. Fruits are 3.7-10 cm long, strap-shaped, glabrous, Seeds are flattened and 1-4-seeded. Wood appears smooth and good polish. The heartwood is golden to dark brown, and sapwood is white to pale brownish. Pods are oblong, flat and thin.

**Microscopical studies**

The important anatomical features observed in the leaves and petioles of Dalbergia sissoo are as follows.

**Petiole:**

- Transverse section of petiole is circular in outline.
- The outer most epidermis is made up of single layer of cells and papillose. Most of the cells elongate to form uniseriate trichome.
- 'U' shaped with strongly incurved ends and collateral vascular bundle surrounded by sclerenchyma fibres seen in the centre of the petiole.

**Midrib:**

- T.S of midrib shows a flat surface on the adaxial side and convexity on the abaxial side.
- Abaxial epidermis is papillose and inner walls are gelatinized. The hypodermal region of adaxial and abaxial epidermis is composed of 2 to 4 rows of collenchyma cells.
- Sclerenchyma fibres are present on the adaxial and abaxial side of the vascular bundles.

**Lamina:**

- ❖ Hypodermis on the upper side is made up of large rectangular parenchyma cells.
- ❖ The palisade tissue is made up of columnar closely packed cells. The spongy tissue is composed of loosely arranged round parenchyma cells.
- ❖ A small crystalline grains or prisms or rod shaped crystals are seen in the mesophyll tissue.
- ❖ The smaller veins of the leaf are vertical and trans current.

**Epidermis in surface view**

- ❖ The adaxial foliar epidermis is made up of polygonal parenchyma cells with straight wall and devoid of stomata and with uniseriate trichomes.
- ❖ The abaxial foliar epidermal cells are polygonal and rubiaceous stomata are present.

**Trichomes:**

- ❖ Trichomes are numerous, uniseriate with a short basal cell with blunt tip.

**Powder microscopy**

The powder microscopy of the leaves showed the following distinct features.

- Epidermal cells with rubiaceous stomata.
- Uniseriate trichomes are noticed.
- Polygonal parenchyma cells are present.
- Sclerenchyma fibres are present.
- Vascular bundles are seen.
- Lignified xylem fibres are abundant in the powder.

**Quantitative microscopy**

Leaf constants such as stomatal number, stomatal index, vein islet and vein termination were determined for the leaves of this plant and the results were tabulated in table 1.

**Stomatal number and Stomatal index**

Leaf surfaces are equipped with small openings or pores called stomata which allow carbon dioxide to enter the leaf and oxygen to escape to facilitate photosynthesis. The number of stomata on leaf surfaces varies widely among different species of plants. Generally, the lower epidermis of the leaf tends to have a higher total than the upper surface. Stomata may also vary in response to the amount of annual rainfall in different localities.

**Vein Islet Number and Vein Termination Number**

Veins provide support for the leaf and transport both water and minerals (via xylem) and food energy (via phloem) through the leaf and on to the rest of the plant. The age differences may not be sufficiently great to influence very materially the size of the vein-islets.

**Palisade Ratio**

Palisade cells are positioned towards the upper surface of the leaf and contain the largest number of chloroplasts per cell in plants. This makes them the primary site of photosynthesis in a plant's leaves. They have a very large surface area in order to absorb more light during photosynthesis.

**Table 1: Quantitative microscopical parameters of the leaf of *Dalbergia sissoo***

| S. No. | Parameters* | Values obtained |
|---|---|---|
| 1 | Stomatal number in upper epidermis | 31.17 ± 0.27 |
| 2 | Stomatal number in lower epidermis | 16.84 ± 1.32 |
| 3 | Stomatal index in upper epidermis | 21.8 ± 0.78 |
| 4 | Stomatal index in lower epidermis | 24.3 ± 0.96 |
| 5 | Vein islet number | 18.5 ± 0.94 |
| 6 | Vein termination number | 5.7 ± 1.79 |
| 7 | Palisade ratio in upper epidermis | 3.40 ± 0.29 |

***mean of three readings** ± SEM

**Table 2: Behavior of the *Dalbergia sissoo* powder with various chemical reagents**

| **Powder + Reagents** | **Colour / Precipitate** | **Presence of active principle** |
|---|---|---|
| Picric acid | Yellow precipitate | Protein present |
| Conc. sulfuric acid | Reddish brown color | Phyto sterols present |
| Lieberman Burchard reagent | Reddish brown color | Phyto sterols present |
| Aqueous ferric chloride | Greenish black color | Tannins present |
| Iodine solution | Blue color | Starch present |
| Mayer's reagent | No cream color | Absence of alkaloids |
| Spot test | No stain | Fixed oils absent |
| Sulfosalicylic acid | White precipitate | Protein present |
| Aq. Sodium hydroxide | Yellow color | Flavanoids present |
| Mg – HCl | Magenta color | Flavanoids present |
| Aq. Lead acetate | White precipitate | Presence of tannins |

**Fluorescence analysis:**

The organic molecules absorb light usually over a specific range of wave length and many of them reemit such radiations. So if the powder is treated with different chemical reagents and seen in the UV chamber, different colours will be produced.

**Table: 3 Fluorescence Analysis of powder of *Dalbergia sissoo***

| **Powder +reagent** | **Day light** | **UV light (254 nm)** | ***UV light (366 nm)*** |
|---|---|---|---|
| Drug powder | Green | Green | Brown |
| Drug powder +aqueous 1M sodium hydroxide | Green | Green | Brown |
| Drug powder + alcoholic 1M sodium hydroxide | Green | Green | Brown |
| Drug powder + iodine | Red | Brown | Brown |
| Drug powder + 10% potassium hydroxide | Yellow | Greenish yellow | Brown |
| Drug powder + 1M hydrochloric acid | Green | Green | Brown |
| Drug powder + glacial acetic acid | Yellow | Greenish yellow | Brown |
| Drug powder + 50% sulphuric acid | Green | Green | Brown |
| Drug powder + 50% nitric acid | Green | Green | Brown |
| Drug powder + 50% hydrochloric acid | Green | Green | Brown |

**Table: 4 Fluorescence Analysis of extracts of *Dalbergia sissoo***

| **Extracts** | **Consistency** | **Colour in Day Light** | **Colour under UV Lamp** | |
|---|---|---|---|---|
| | | | **360 nm** | **254nm** |
| Petroleum extract | Semisolid | Yellow | Orange | Yellow |
| Hexane extract | Semisolid | Yellow | Reddish orange | Green |
| Ether extract | Semisolid | Greenish brown | Green | Greenish brown |
| Chloroform extract | Semisolid | Yellowish brown | Orange | Yellowish brown |
| Ethanol extract | Semisolid | Yellowish green | Orange | Green |
| Methanol extract | Semisolid | Yellowish green | Orange | Green |
| Aqueous extract | Semisolid | Brown | Green | Dark green |

Therefore it can be used for the identification of the drug. The fluorescence characteristic of the drug powder with different chemical reagent was studied by observing under UV Light at 254nm and 365 nm. The tests and observations are recorded. It suggested

that a non-fluorescent compound may fluorescent if it mixed with impurities. Therefore, the results obtained from the present fluorescent studies will also help to check any impurities present in leaf powder of *D.sissoo*

**Loss on drying:**

The loss on drying of plant materials should be determined and the water content should also be controlled. This is especially important for materials that absorb the moisture easily or deteriorate quickly in presence of water. The test determines both water and volatile matter.

**Foreign matter:**

Drugs should be free from moulds, insects, animal faecal matter and other contaminations such as earth, stones and extraneous material. The amount of foreign matter shall not be more than the percentage prescribed in the monograph.

**Ash value:**

The residue remaining left after incineration of the crude drug is called as ash. The residue obtained usually represents the inorganic salts that are naturally occurring in the drug and adhering to it. It varies with in definite limits according to the soils. It may also include inorganic matters deliberately added for the purpose of adulteration. Hence, an ash value determination furnishes the basis for judging the identity and cleanliness of any drug and gives information relative to its adulteration or contamination with inorganic matter, thus ash values are helpful in determining the quality and purity of drug.

The total ash of a crude drug reflects the care taken in its preparation. The acid insoluble ash is a part of the total ash that is insoluble in dilute hydrochloric acid. A higher limit of acid-insoluble ash is imposed, especially in cases where silica may be present or when

the calcium oxalate content of the drug is higher. Water-soluble ash is the water soluble portion of the total ash; these ash values are important quantitative standards.

**Table: 5 Analytical parameters of *D.sissoo***

| S. No | Parameters* | Values* expressed as % |
|---|---|---|
| **1.** | **Volatile oil** | 0.2mL |
| **2.** | **Foreign organic matter** | 0.01 ± 0.12 |
| ***3.*** | **Moisture content** | 7.88 ± 0.01 |
| ***4.*** | **Ash values** | |
| | Total ash | 8.43 ± 0.49 |
| | Acid insoluble ash | 3.33 ± 0.97 |
| | Water soluble ash | 4.2±0.90 |
| | Water insoluble ash | 7.8 ± 0.91 |

***mean of three readings** ± SEM

**Extractive values:**

It determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material.

Ethanolic extract(70%) showed highest extractive value (28.74 %). When compared to ethanolic extract(95%) (22.78%), chloroform extract (4.88%),petroleum ether extract (8.86%) and the results were presented in table 6.

**Foaming index:**

Foaming index is less than 100 which indicate absecnce of saponins in leaves which finally proves by phytochemical screening table 6.

**Swelling index:**

Many medicinal plant materials are of specific therapeutic or pharmaceutical utility because of their swelling properties, especially gums and those containing an appreciable amount of mucilage, pectin or hemicellulose.

**Table: 6 Analytical parameters – Extractive values of *D.sissoo***

| S. No | Parameters* | Values* expressed as % |
|---|---|---|
| ***1.*** | **Extractive Values** | |
| | Petroleum extract | 8.86± 0.48 |
| | Hexane extract | 7.81 ± 0.91 |
| | Ether extract | 9.82± 0.02 |
| | Chloroform extract | 4.88± 0.04 |
| | Ethanol extract | 22.78 ±0.81 |
| | 70 % Ethanol extract | 28.74 ±0.71 |
| | Methanol extract | 21.47 ± 0.01 |
| | Aqueous extract | 18.94± 0.74 |
| ***2.*** | **Foaming index** | less than 100 |
| ***3.*** | **Swelling index** | **expressed as mL** |
| | Initial volume | 3.6 ± 0.20 |
| | Final volume | 10.3 ± 0.97 |

***mean of three readings** ± SEM

**PLANT TISSUE CULTURE:**

Tissue culture of plant tissues is a method that has a wide variety of applications, including micropropagation, embryogenesis, organogenesis, and callus culture. The induction of callus, which is a cluster of undifferentiated cells, begins with a small section of plant tissue or explant that is manipulated using plant growth regulators to induce the production of calli.

Leaf disc was cultured on MS basal medium and it supplemented with various levels of BAP and NAA. It was observed 100% in MS medium supplemented with different concentrations of BAP and NAA. In this 3.0 mg/L BAP + 0.5 mg/L NAA combination produced highest fresh weight of callus per culture and it was 10.3 g. The response from the explants took 18 days old culture in the same media compositions. The colour of callus was green and friable in nature in this case. The second best 90% of callus was found in 2.5 mg/L BAP + 0.5 mg/L IAA. The calli were friable in nature. The present findings are almost similar to that of M.A. Bari's (2008) result. But according to M.A. Bari's the100% response was observed at MS medium supplemented with 2.0 mg/1 BAP + 0.5 mg/1 NAA.

**Table: 8 Concentrations of Plant growth hormones**

| S.No | Hormone concentration | Callus growth |
|---|---|---|
| 1 | Basal Medium (BM) | - |
| 2 | BM+ BAP 0.5 mg /L + NAA 0.5 mg /L | + |
| 3 | BM+ BAP 1mg /L + NAA 0.5 mg /L | + |
| 4 | BM+ 1.5mg BAP/L + NAA 0.5 mg /L | + |
| 5 | BM+ BAP 2 mg /L + NAA 0.5 mg /L | + |
| 6 | BM+ BAP 2.5mg /L + NAA 0.5 mg /L | ++ |
| 7 | BM+ BAP 3mg /L + NAA 0.5 mg /L | +++ |
| 8 | BM+ BAP 3.5mg /L + NAA 0.5 mg /L | + |
| 9 | BM+ BAP 4mg /L + NAA 0.5 mg /L | + |

- No response + Poor callus ++ Better growth
+++ Good growth

The aim was to increase the production and analysis of secondary metabolites from the cultured callus of *D. sisoo* using the above prescribed methods for the possible

physiological functions so their significance in pharmacology must be very high. Hence our results prove that the modern tissue culture technique is rapid, inexpensive method to isolate high concentration and disease resistance secondary metabolite for effective treatment of ailments.

The secondary plant metabolites are synthesized via multi-step enzymatic pathways in which several genes are involved. Thus it is not very easy to try to regulate such systems. The chemical synthesis of many plant-derived metabolites is often not feasible, and it is possible that in the future most of them have still to be isolated from plant material. For this purpose callus induction of medicinal plants might offer a useful alternative source to practice tissue culture by different industries, such as the horticultural, agricultural, and pharmaceutical industries. Since plant cells are totipotent, using cells to produce new plantlets or its products can shorten the time needed by eliminating the need to wait until a plant or tree matures.

In general, tissue growth and the quality of callus responses are strongly influenced by the type and concentrations of nutrients present in the culture media. Effect of different macronutrient formulations on number and growth of in vitro cultured explants were investigated by many workers and reported that some species give analogous response in all media while others show preference for a specific medium for explant establishment and growth. The results obtained in the present study clearly indicated that the response of leaf explants was dependent on the origin of the explants.

On observations it was found that the MS medium without any growth regulator failed to elicit regeneration, but addition of BAP and NAA proved to be significantly useful in callus production of *D. sissoo*. So the *in vitro* propagation protocols developed in the present study

can be effectively utilized for producing secondary metabolite for better pharmacological action of *D. sissoo*.

**PHYTOCHEMICAL STUDIES:**

Collection, authentication, preparation and isolation of active component from *D.sissoo* leaves have been discussed in this chapter. The various extracts of powdered leaf materials and ethanolic extract of callus were subjected to preliminary phytochemical screening, estimation and isolation of active principle for the discussed aim.

**Preliminary phyto chemical analysis:**

Preliminary phyto chemical analysis was performed for different extracts and the results were tabulated in table 9 & 10.

**TABLE: 9**

**PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE LEAF POWDER OF *Dalbergia sissoo***

| S.NO | TEST | RESULTS |
|---|---|---|
| **1.** | **TEST FOR STEROLS** | |
| | a. Salkowski's test | + |
| | b. Libermann- burchard's test | + |
| **2.** | **TEST FOR CARBOHYDRATES** | |
| | a. Molisch's test | + |
| | b. Fehling's test | + |
| | c. Benedict's test | + |
| **3.** | **TEST FOR PROTEINS** | |
| | a. Millon's test | + |
| | b. Biuret test | + |
| **4.** | **TEST FOR ALKALOIDS** | |
| | a. Mayer's reagent | - |
| | b. Dragendroff's reagent | - |
| | c. Hager's reagent | - |
| | d. Wagner's reagent | - |
| | e. Test for Purine group (Murexide test ) | - |
| **5.** | **TEST FOR GLYCOSIDES** | |
| | a. Anthraquinone glycosides | |
| | i) Borntrager's test | + |
| | ii) Modified Borntrager's test | + |
| | b. Cardiac glycosides | |
| | i) Keller Killiani test | + |
| | c. Cyanogenetic glycosides | - |
| **6.** | **TEST FOR SAPONINS** | - |
| **7.** | **TEST FOR TANNINS** | |
| | $Fecl_3$ test | + |
| **8.** | **TEST FOR FLAVONOIDS** | |
| | a. Shinoda test | + |
| | b. Alkali test | + |
| | c. Acid test | + |
| **9.** | **TEST FOR TERPENOIDS** | + |
| **10.** | **TEST FOR VOLATILE OILS** | + |
| **11.** | **TEST FOR MUCILAGE** | + |

**(+) indicates positive reaction (-) indicates negative reaction**

**Table: 10 PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE VARIOUS EXTRACTS OF LEAF POWDER OF *Dalbergia sissoo***

| S. No. | Chemical Test | Hexane extract | Petroleum ether extract | Ether extract | chloroform | Methanol extract | Ethanol extract | Aqueous extract |
|---|---|---|---|---|---|---|---|---|
| 1. | Terpenoids | + | + | + | + | + | + | - |
| 2. | Flavones | - | - | - | - | + | + | + |
| 3. | Steroids | + | + | + | + | + | + | - |
| 4. | Anthraquinones | - | - | - | + | + | + | + |
| 5. | Glycosides | - | - | - | - | + | + | - |
| 6. | Sugars | - | - | - | - | + | + | + |
| 7. | Alkaloids | - | - | - | - | - | - | - |
| 8. | Quinones | - | - | - | - | - | - | - |
| 9. | Phenols | - | - | - | - | + | + | + |
| 10. | Tannins | - | - | - | - | + | + | + |
| 11. | Saponins | - | - | - | - | - | - | - |
| 12. | Proteins & free amino acids | - | - | - | - | + | + | + |

**(+) indicates positive reaction (-) indicates negative reaction**

**Table:11 PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE ETHANOLIC EXTRACT OF CALLUS OF *Dalbergia sissoo***

| S.NO | TEST | RESULTS |
|---|---|---|
| 1. | Test for sterols | - |
| 2. | Test for carbohydrates | + |
| 3. | Test for flavonoids | + |
| 4. | Test for proteins | + |
| 5. | Test for alkaloids | - |
| 6. | Test for glycosides | + |
| 7. | Test for saponins | - |
| 8. | Test for tannins | + |

**(+) indicates positive reaction (-) indicates negative reaction**

**Estimations:**

The both leaf and callus extracts were estimated quantitatively for total phenolic, total tannin and total flavonoid content.

**Total Phenolic Content:**

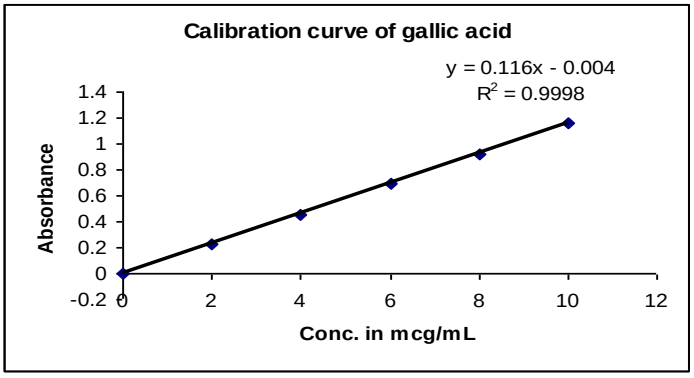
In the present study, total phenolic content present in extract was estimated using modified Folin- ciocalteau method. Values are expressed as gallic acid equivalents and the results were tabulated.Total phenolic content for DSEE and ethanolic extract of calli of *Dalbergia sissoo* were found to be 88.98±3.0 mg/g and 125.12±1.8 mg/g respectively.

**Table 12: Total phenolic content in ethanolic extract of *D. sissoo* and ethanolic extracts of *Dalbergia sissoo* from callus in terms of Gallic acid equivalents**

| S. No. | Conc. of gallic acid in µg/mL | Absorbance at 760nm | Conc. of ethanolic extract in µg/mL | Absorbance at 760nm | | Amount of total phenolic content in terms mgGAE/g of extract | |
|---|---|---|---|---|---|---|---|
| | | | | DSEE | DSEEC | | |
| | | | | | | DSEE | DSEEC |
| 1 | 20 | 0.229± 0.010 | 50 | 0.535±0.006 | 0.735±0.011 | 92.75±1.4 | 127.41±0.3 |
| 2 | 40 | 0.452± 0.006 | 100 | 0.984±0.006 | 1.421±0.009 | 85.22±0.5 | 122.84±0.8 |
| 3 | 60 | 0.695± 0.005 | | | | | |
| 4 | 80 | 0.918± 0.031 | | | | | |
| 5 | 100 | 1.162± 0.028 | | | | | |
| | | | | | **Average** | **88.98±3.0** | **125.12±1.8** |

***mean of three readings** ± SEM

**Fig. 6.1: Calibration graph of gallic acid**




The linear regression equation was found to be y=0.116x-0.004 while the correlation was found to be 0.9998. The amount of phenolic content present in the extract and extract

from callus in terms mg GAE/g of extract were found to be 88.98 ± 3.07 and 125.12±1.8by using the above linear regression equation.

Phenolics are the most wide spread secondary metabolite in plant kingdom. These groups of compounds have much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers. The results shows that the ethanolic extract of callus of *Dalbergia sissoo* having more phenolic content than leaf extracts which produce an additive effect in reduction of obesity greatly.

**Estimation of Flavonoid:**

Flavonoid contents were determined by colorimetric method using $AlCl_3$. The flavones and flavonols react and form more stable complexes with Aluminium chloride. The amount of flavonoid was considered as the important index for evaluating the biological activity of all material.

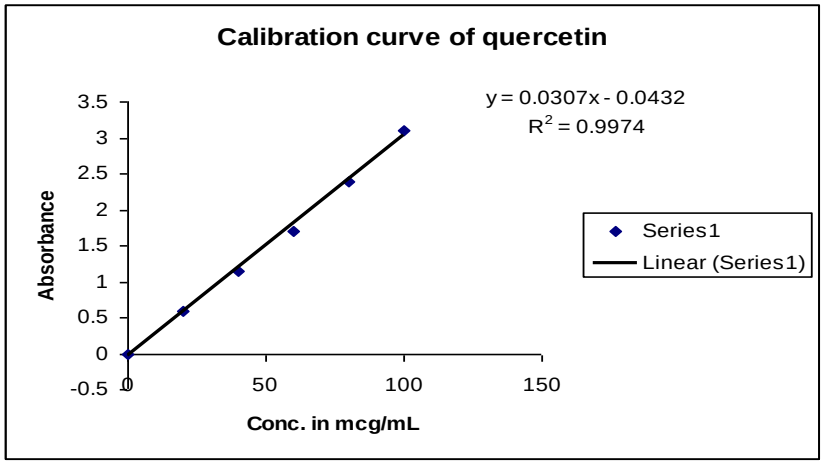
Total flavonoid content for ethanolic extract of leaves of *Dalbergia sissoo* and ethanolic extract of calli of *Dalbergia sissoo* were found to be **47.64±1.8 mg/g and 82.53±0.5 mg/g** respectively. The linear regression equation was found to be y = 0.0307x - 0.0432 while the correlation was found to be 0.9974. The amount of flavonoid content present in the extract and extract from callus in terms mg quercetin /g of extract were found to be **47.64±1.83** and **82.53±0.5** by using the above linear regression equation.

**Table: 13 Total flavanoid content in ethanolic extract of *D. sissoo* and ethanolic extracts of *Dalbergia sissoo* from callus in terms of quercetin equivalents**

| S.No | Conc. of quercetin in µg/mL | Absorbance at 415nm | Conc. of ethanolic extract in µg/mL | Absorbance at 415nm* | | Amt of total flavonoid content in terms mg quercetin equivalent/ g of extract* | |
|---|---|---|---|---|---|---|---|
| | | | | DSEE | DSEEC | DSEE | DSEEC |
| 1 | 20 | 0.589 ± 0.01 | 100 | 0.109± 0.06 | 0.281± 0.03 | 49.89±0.2 | 81.82±0.9 |
| 2 | 40 | 1.151 ± 0.04 | 200 | 0.231 ± 0.01 | 0.468 ± 0.01 | 45.4±1.4 | 83.25±1.4 |
| 3 | 60 | 1.710 ± 0.09 | | | | | |
| 4 | 80 | 2.390 ± 0.03 | | | | | |
| 5 | 100 | 3.112 ± 0.03 | | | | | |
| | | | | | Average | **47.64±1.8** | **82.53±0.5** |

***mean of three readings** ± SEM

**Fig. 6.2: Calibration curve of Quercetin**




Flavonoids are the most diverse group of polyphenols and are consist of a basic $C_6$-$C_3$-$C_6$ flavone skeleton. Six classes of flavonoids are widespread in higher plant, and include the chalcones, flavanones, flavandiols, flavones, anthocyanins, catechins, and condensed tannins. Flavonoids play roles in plants, such as pigmentation, protection against UV-radiation, reproduction, and regulation of plant growth.. Flavonoids have significantly higher ROS scavenging activity, as compared to vitamin C and carotenoids . Its antioxidative activity is dependent on the chemical structure, such as the number of hydroxyl groups substituted on

the B ring. Intake of flavonoids has been associated with reduced incidences of cancer, heart disease, and various neurological disorders.

**Total Tannin Content:**

Total tannins determination is carried out by spectrophotometry after oxidation of the analyte with the Folin–Denis reagent in alkaline medium. This method is based on a redox reaction and other reducing agents in the samples.

Total tannin content for ethanolic extract of leaves of *Dalbergia sissoo* and ethanolic extract of calli of *Dalbergia sissoo* were found to be 281.01 mg/g and 413.09 mg/g respectively.

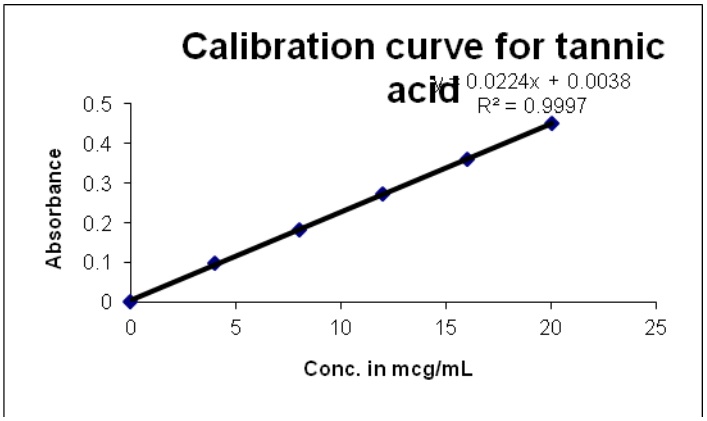
**Table: 14 Total tannin content in ethanolic extract of *D. sissoo* and ethanolic extracts of *Dalbergia sissoo* from callus in terms of Tannic acid equivalents**

| S.No. | Conc. of Tannic acid in µg/mL | Absorbance at 700nm | Conc. of ethanolic extract in µg/mL | *Absorbance at 700nm | | *Amt of total Tannin content in terms mg tannic acid equivalent/ g of extract | |
|---|---|---|---|---|---|---|---|
| | | | | DSEE | DSEEC | DSEE | DSEEC |
| 1 | 20 | 0.589 ± 0.01 | 20 | 0.138± 0.001 | 0.197± 0.011 | 281.01±3.96 | 413.09±4.8 |
| 2 | 40 | 1.151 ± 0.04 | | | | | |
| 3 | 60 | 1.710 ± 0.09 | | | | | |
| 4 | 80 | 2.390 ± 0.03 | | | | | |
| 5 | 100 | 3.112 ± 0.03 | | | | | |
| | | | | **Average** | | **281.01** | **413.09** |

***** mean of three readings ± SEM

The linear regression equation was found to be y = 0.022x + 0.003 while the correlation was found to be 0.999. The amount of tannin content present in the ethanolic extract of *D.sissoo* and ethanolic extracts of *D. sissoo* from callus in terms mg Tannic acid/g of extract was found to be **281.01 and 413.09** by using the above linear regression equation.

**Fig-6.3: Calibration curve for Tannic acid**




It is known that plant tannin can be precipitated by many chemical reagents and these precipitation techniques have become the tool for the estimation. Tannins in high concentrations reduce intake, digestibility of protein and carbohydrates. Tannins in low to moderate concentrations prevent bloat and increase the flow of non-ammonia nitrogen and essential amino acids from the rumen. Tannins may reduce cell wall digestibility by binding bacterial enzymes and (or) forming indigestible complexes with cell wall carbohydrates .The results increase the pharamacological potency of the extract for the anti obesity activity.

**Plant tissue culture Vs Traditional approach:**

The total phenolic, flavonoid, tannins content in leaf-derived callus and *in vivo* leaf tissues of *D.sissoo* were compared to elucidate the better therapeutic potential of the extract from the modern approach for drug development.

**Table: 15 Comparison Plant Tissue Culture and In vivo Plant Extract**

| Name of the Extract | Amt of total phenolic content in terms mgGAE/g of extract | Amt of total flavonoid content in terms mg quercetin equivalent/ g of extract | Amt of total Tannin content in terms mg tannic acid equivalent/ g of extract |
|---|---|---|---|
| **Ethanolic extract of leaves of *Dalbergia sissoo*** | 88.98 | 47.64 | 281.01 |
| **Ethanolic extract of calli of *Dalbergia sissoo*** | 125.12 | 82.53 | 413.09 |

The ethanolic extract of callus exhibited highest phenolic content, flavonoid content and tannin content when compared to ethanolic extract of leaves. This study indicates the significance of callus induction and confirms the aim of the present study and believed that it will provide a new way for the pharmaceutical industries for production of potent and efficacious herbal drug development in near future.

**Isolation of active principle:**

Metabolomic approach of our study is to identify the plant active constituent using the analytical procedures and to estimate their pharmacological properties for the potent phytomedicine development.

The early fractions (60-71), obtained after elution with Chloroform: methanol (90:10) showed single spot in chloroform: methanol (9:1) solvent system. The chemical test showed positive for flavonoids.

**Table: 17 Chemical tests for the isolated fraction**

| S.NO | TEST | RESULTS |
|---|---|---|
| **1.** | **TEST FOR STEROLS** | |
| | a. Salkowski's test | - |
| | b. Libermann- burchard's test | - |
| **2.** | **TEST FOR CARBOHYDRATES** | |
| | a. Molisch's test | - |
| | b. Fehling's test | - |
| **3.** | **TEST FOR PROTEINS** | |
| | a. Millon's test | - |
| | b. Biuret test | - |
| **4.** | **TEST FOR ALKALOIDS** | |
| | a. Mayer's reagent | - |
| | b. Dragendroff's reagent | - |
| | c. Hager's reagent | - |
| | d. Wagner's reagent | - |
| **5.** | **TEST FOR GLYCOSIDES** | |
| | a. Anthraquinone glycosides | |
| | i) Borntrager's test | - |
| | ii) Modified Borntrager's test | - |
| | b. Cardiac glycosides | |
| | i) Keller Killiani test | - |
| | c. Cyanogenetic glycosides | - |
| **6.** | **TEST FOR SAPONINS** | |
| **7.** | **TEST FOR TANNINS** | |
| | $Fecl_3$ test | - |
| **`8.** | **TEST FOR FLAVONOIDS** | |
| | a. Shinoda test | + |
| | b. Alkali test | + |
| | c. Acid test | + |

The melting point was found to be 255$^{o}$C. The EI MS spectrum showed a molecular ion peak at m/z 341.7 in addition to the peaks at m/z 313 (M+-CO), m/z 264, 209.

In UV analysis it shows the λ max in methanol at 315, 258nm showed the characteristic absorption of isoflavone. No UV bathochromic shift was observed with $AlCl_3$ suggesting the absence of free 5 OH group and a bathochromic shift was observed at 353, 271 nm upon the addition of potassium acetate. It indicates the presence of 7 OH group. The $^{1}H$

NMR datas are tabulated and from the previous literature the isolated isoflavonoid was identified as dalpatein which was previously isolated from the flowers and leaves of *Dalbergia paniculata*. This is a first time dalpatein has been reported from the leaves of *D. sissoo*

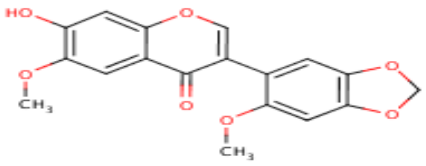


**Table 18: Phytochemical evaluation of ethanolic extract and isolated fractions by TLC studies.**

| S.No | Solvent System | Name of Extract | No. of spots | Rf value | Detecting Agent |
|---|---|---|---|---|---|
| 1. | Chloroform: Methanol 9:1 | DSEE | 1 | 0.97 | UV 366 nm |
| | | | 2 | 0.91 | |
| | | | 3 | 0.81 | |
| | | | 4 | 0.75 | |
| | | | 5 | 0.62 | |
| | | | 6 | 0.44 | |
| 2. | | DSIF | 1 | 0.80 | |
| 3. | Ethyl acetate: Formic acid: Glacial acetic acid: Water 100:11:11:26 | DSEE | 1 | 0.94 | |
| | | | 2 | 0.87 | |
| | | | 3 | 0.80 | |
| | | | 4 | 0.74 | |
| | | | 5 | 0.41 | |
| | | | 6 | 0.28 | |
| 4. | | DSIF | 1 | 0.79 | |

**Table: 19 Interpretation of IR**

| S.No. | FREQUENCY ($cm^{-1}$) | GROUPS ASSIGNED |
|---|---|---|
| 1 | 3402.36 | May be due to aromatic OH stretching |
| 2 | 2929.48 | May be due to aliphatic C-H stretching |
| 3 | 2345.78 | May be due to C=C stretching |
| 4 | 1586.04 | May be due to C=0 stretching. |
| 5 | 1424.27 | May be due to C-H bending |
| 6 | 1019.92 | May be due to C-O stretching |

**Table No: 20 Interpretation of NMR**

| S.No | Ppm (δ Value) | Groups assaigned |
|---|---|---|
| 1. | 8.039 | May be due to aromatic OH group |
| 2. | 7.989, 7.468 | May due to aromatic proton |
| 3. | 6.998 | May be due to methine group (CH) |
| 4. | 3.867 | May be due $OCH_3$ group |
| 5. | 2.50 | May be due to $CH_2$ group |

**PHARMACOLOGICAL SCREENING**

**Anti-oxidant activity:**

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves.

**Table: 21 Comparison of $IC_{50}$ Value for antioxidants**

| Methods | $IC_{50}$ (µg/mL) | | | |
|---|---|---|---|---|
| | **Ascorbic acid** | **Ethanolic extract of *Dalbergia sissoo*** | **Dalpatein** | **Ethanolic extract of callus of *Dalbergia sissoo*** |
| **$H_2O_2$ Scavenging** | 24.48 | 32.74 | 26.88 | 28.69 |
| **DPPH assay** | 19.69 | 142.44 | 70.29 | 98.41 |
| **Total anti oxidant assay** | 64.21 | 77.64 | 73.10 | 71.62 |

Reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$), singlet oxygen ($O_2$), hydroxyl radical (·OH) and superoxide ($O_2$.-), are produced in the respiratory chain in the mitochondria and are byproducts of normal cellular metabolism (Finkel and Holbrook 2000). Perturbed metabolism leads to accumulation of toxic levels of ROS, and oxidative stress results when the rate of generation of ROS exceeds the rate of their disposal (Hodges et al. 2004). Both biotic (e.g. pathogens) and abiotic factors (e.g. extreme temperature, light, storage duration, processing methods, and conditions accelerating water loss or/and ripening) can trigger excess production of ROS under growth and post-harvest conditions (Hodges 2003). Excessive ROS can react with various cellular components such as lipids, carbohydrates, proteins and nucleic acids.

**Phenolics as free radical scavengers:** Phenolic compounds (POH) act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (R):

$$\mathbf{R + POH \rightarrow RH + PO^{\cdot}}$$

The phenoxy radical intermediates ($PO^{\cdot}$) are relatively stable due to resonance and therefore a new chain reaction is not easily initiated. Moreover, the phenoxy radical intermediates also act as terminators of propagation route by reacting with other free radicals:

$$\mathbf{PO^{\cdot} + R^{\cdot} \rightarrow POR}$$

Phenolic compounds possess ideal structure chemistry for free radical scavenging activities because they have: (1) phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; (2) extended conjugated aromatic system to delocalize an unpaired electron.

Flavonols are particularly reactive toward ROS because of their peculiar chemical structure of electron deficiency.

**Hydrogen peroxide scavenging activity assay:**

The *D. sissoo* also demonstrated for hydrogen peroxide decomposition activity in a concentration dependent manner. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, $H_2O_2$ can probably react with Fe2+, and possibly Cu2+ ions to form hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of $H_2O_2$ by *D.sissoo* may at least partly result from its antioxidant and free radical scavenging activity.

$$H_2O_2 + Cu+ \text{ or } Fe^{2+} \rightarrow Cu^{2+} \text{ or } Fe^{3+} + OH + {\cdot}OH-$$

The scavenging of hydrogen peroxide by phenolic compounds has been attributed to their electron-donating ability. With different concentrations of crude and ethanolic extracts high electron-donating abilities and scavenging activity was achieved. In comparison, the $IC_{50}$ and percentage inhibition of hydrogen peroxide scavenging activity was tabulated.

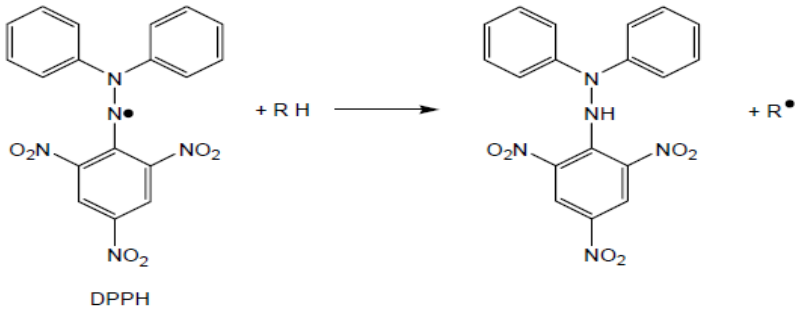
**Table: 22 Percentage inhibition of hydrogen peroxide of *Dalbergia sissoo***

| S. No. | Conc. in µg/mL | Percentage inhibition | | | |
|---|---|---|---|---|---|
| | | **Ascorbic acid*** | **Ethanolic extract of *Dalbergia sissoo**** | **Dalpatein*** | **Ethanolic extract of callus of *Dalbergia sissoo**** |
| 1 | 10 | 36.4±3.46 | 24.78±2.12 | 32.58±2.48 | 29.48±3.48 |
| 2 | 20 | 45.91±1.25 | 32.04±3.46 | 49.36±3.12 | 41.58±2.15 |
| 3 | 30 | 52.17±3.59 | 40.05±1.29 | 52.89±1.48 | 48.47±4.72 |
| 4 | 40 | 79.58±2.28 | 64.58±2.54 | 69.87±3.08 | 67.95±2.17 |
| 5 | 50 | 91.23±3.31 | 73.21±3.31 | 78.94±1.98 | 79.69±4.12 |
| | **$IC_{50}$** | **24.48µg/mL** | **32.74µg/mL** | **26.88µg/mL** | **28.69 µg/mL** |

* mean of three readings± SEM

***DPPH radical scavenging activity:***

The scavenging of the DPPH radical by hydrogen donating antioxidants is characterized by a rapid decline in the absorbance at 517 nm. The rapid reaction between antioxidants and DPPH occurs with the transfer of the most labile H atoms to the radical, while the subsequent slow step depends on the residual H-donating capacity of antioxidant degradation products. The antioxidants react with DPPH and convert it to 1, 1-diphenyl-2-picryl hydrazine with decolouration (from deep violet to light yellow).




Ascorbic acid is used as standard that exhibits a very rapid initial step, and the disappearance of the purple colour of DPPH occurs almost immediately upon contact between reactants. The radical scavenging activity of ascorbic acid, crude ethanolic extract, isolated compound and ethanol extracts of callus increases in a dose-dependent manner and the results were tabulated.

**Table: 23 Percentage inhibition of DPPH of *Dalbergia sissoo***

| S. No. | Conc. in µg/mL | Percentage inhibition | | | |
|---|---|---|---|---|---|
| | | **Ascorbic acid*** | **Ethanolic extract of Dalbergia sissoo*** | **Dalpatein *** | **Ethanolic extract of callus of *Dalbergia sissoo**** |
| 1 | 40 | 91.06±0.83 | 16.03±0.26 | 56.43±0.18 | 32.41±0.86 |
| 2 | 80 | 93.27±1.56 | 23.57±3.49 | 67.58±1.49 | 45.37±0.02 |
| 3 | 120 | 94.77±0.91 | 33.89±2.14 | 73.43±1.37 | 61.29±0.97 |
| 4 | 160 | 95.58±2.54 | 56.2±2.69 | 80.05±2.71 | 75.29±1.27 |
| 5 | 200 | 96.38±1.89 | 77.12±1.47 | 89.17±0.97 | 89.92±1.46 |
| | **$IC_{50}$** | **19.69µg/mL** | **142.44µg/mL** | **70.29 µg/mL** | **98.41µg/mL** |

* mean of three readings± SEM

**The total antioxidant capacity:**

Free radical scavenging of phenolic compounds is an important property underlying their various biological and pharmacological activities. The Phosphomolybdenum assay is based on the reduction of Mo (VI) to Mo (V) by antioxidant compounds and a formation of green phosphate/ Mo(V) complex with a maximal absorption at 695 nm and it was efficient to extend its application to plants polyphenols. Total antioxidant capacity of ascorbic acid, ethanolic extract of leaves of *D.sissoo*, dalpatein and ethanolic extract of callus were tabulated.

**Table: 24 Total Anti oxidant assay of *Dalbergia sissoo***

| S. No. | Conc. in µg/mL | Percentage inhibition | | | |
|---|---|---|---|---|---|
| | | **Vitamin C*** | **Ethanolic extract of Dalbergia Sissoo*** | **Dalpatein*** | **Ethanolic extract of callus of *Dalbergia sissoo**** |
| 1 | 40 | 57.69±1.27 | 49.57±0.94 | 53.87±2.47 | 51.97±1.41 |
| 2 | 80 | 68.57±2.98 | 57.81±1.57 | 61.47±1.04 | 63.57±2.17 |
| 3 | 120 | 75.75±1.92 | 69.14±2.01 | 71.75±0.07 | 69.58±0.043 |
| 4 | 160 | 85.14±2.91 | 78.67±1.37 | 81.94±1.79 | 80.47±1.37 |
| | **$IC_{50}$** | **64.21µg/mL** | **77.64 µg/mL** | **73.10 µg/mL** | **71.62µg/mL** |

* mean of three readings± SEM

These studies showed that ethanolic extract of leaves of *D.sissoo*, Isolated compound and ethanolic extract of callus exhibited significant radical scavenging activity.

***In vitro* pancraetic lipase inhibition** [184-188]

One of the important strategies in the treatment of obesity includes the development of inhibitors of nutrient digestion and absorption, in an attempt to reduce the energy intake through gastrointestinal mechanisms, without altering any central mechanisms. Pancreatic lipase inhibition is one of the most widely studied mechanisms used to determine the potential efficacy of natural products as antiobesity agents.

The phytochemical screening of *Dalbergia sissoo* shows the presence of flavonoid, glycosides, tannins, terpenes, phenols and saponins. Our finding is to indicate that *Dalbergia sissoo* inhibits pancreatic lipase. The inhibitory activities of different concentrations of standard and tests compounds such as ethanolic extract of leaves of *D.sissoo* and isolated compound were tested against chicken pancreatic lipase using olive oil as the substrate. The activity of lipase was affected when incubated with the standard and test compounds. The $IC_{50}$ values of the orlistat, DSEE and isolated compound were calculated from the least regression fit to be **2.049±0.98, 3.89±0.97** and **2.39±0.43**respectively. The dose dependent pancreatic lipase inhibitory activity was observed. i.e., inhibition of enzyme was increased on increasing the concentration of extract.

Flavonoid and their antioxidative metabolites [196] have the potential to be obesity-preventive agents in the following *Alpinia officinarum*[197, 198]*Taraxacum officinale*[199], *Actinidia arguta, Aesculus turbinate, Arachis hypogaea* reduce the TG plasma level and body weight gain. [200] *Camellia sinensis* reduce Body weight gain and visceral fat. *Eleutherococcus senticosus* decrease abdominal fat, TG in liver and serum and LDL in serum. *Humulus lupulus* decreases body weight gain and blood glucose levels.

A number of plants and natural products have been screened for their pancreatic lipase inhibitory activity but just some of them have gone up to clinical studies. [194, 195] Orlistat is currently in clinical use, although others are under investigation. Some of them are *Panax ginseng* [201], *Camellia sinensis, Eleutherococcus senticosus, Malusdo mestica* and *Arachis hypogaea*. Some plant extracts show 40 % *in vitro* anti lipase activity such as *Levisticum officinale, Quercu sinfectori, Rosmarinus officinali*], *Vaccinium myrtillus,* and *Glycyrrhiza uralensis*. *Dalbergia sissoo* inhibited pancreatic lipase, it might affect fat absorption and the uptake of fatty acids in the periphery, if enough of the active components can be absorbed and enter the circulation. The clinical effect of orlistat showed that, the reducing the absorption of fat may be an extra effect to dieting in obese patients. Orlistat is a prescription medication available as a pancreatic lipase inhibitor that is not absorbed and, hence, works only within the intestine. Orlistat reduces the absorption of dietary fat by about 30% in adults. Long-term clinical trials have shown that Orlistat results in about 5% greater weight loss. [13]In our study also, a marked inhibition of chicken pancreatic lipase was observed.

**Table: 25 Pancreatic lipase inhibition of *D.sissoo***

| S. No | Concentration (mg/ml) | Percentage Inhibition (%) | | |
|---|---|---|---|---|
| | | Orlistat* | DSEE* | Dalpatein* |
| 1 | 0.5 | 7.14±0.49 | 2.80±1.03 | 5.70±1.34 |
| 2 | 1.0 | 14.28±1.46 | 10.00±0.92 | 17.14±1.05 |
| 3 | 1.5 | 38.57±0.001 | 21.40±1.23 | 34.28±0.78 |
| 4 | 2.0 | 55.71±2.13 | 30.00±0.56 | 51.42±0.97 |
| 5 | 2.5 | 65.71±0.12 | 32.85±1.06 | 52.85±0.37 |
| 6 | 3.0 | 68.57±0.63 | 34.28±0.58 | 55.71±0.07 |
| **IC 50** | | **2.049±0.98** | **3.89±0.97** | **2.39±0.43** |

* mean of three readings± SEM

**Evaluation of the toxic effects of *Dalbergia sissoo* in zebra fish (*Danio rerio*) embryos:**

Zebrafish is a powerful animal model for biological research. Its use ranges from toxicology, developmental biology, biomedicine, neurophysiology, drug discovery model for human diseases [203]. The main advantages of its use, such as the small size and maintenance cost, the transparency of embryos and larvae, and the speed at which these develop ex utero zebrafish has been suited for large throughput screening for drug discovery, including those from natural sources [204].

**Determination of $LC_{50}$**

In this study, the $LC_{50}$ analysis was carried out based on OECD guideline for fish embryo toxicity test (FET) with control group (DMSO treated) for 24, 48 and 72 h periods and the calculation was carried out for 72 h [205].

The dalpatein and crude plant extracts (10 embryos /well) of the concentrations (1, 10, 100µg/L and 1, 10mg/L) in 24-well multi-plates with six triplicates. The embryotoxic effect of Dalpatein and ethanolic extract of leaves of *D.sissoo* on the development of zebrafish embryo determines the $LD_{50}$ were found to be **100mg and 10mg** for embryos respectively. The developmental defects caused by those treatments include bent or hook-like tails, coagulation of eggs, lack of heart rate and lack of somite formation.

The survival rate of zebrafish embryos treated with various concentrations of dalpatein and ethanolic extract (DMSO, Water, **1, 10, 100µg/L and 1, 10mg/L**) were noticed. A significantly decreasing survival rate was observed in when the dose is higher.

According to probit analysis, no mortality was observed in both the water and DMSO medium. It was observed that there was no mortality in 1µl/L and 10µl/L concentration. But

10%, 30% and 40% was observed at 100µl/L, 1mg/ L and 10mg/ L concentration respectively for dalpatein. For DSEE showed 20% in 100µl/L, 40% in 1mg/ L and 50% in 10mg/ L using Schneider-Orelli's formula.

As a natural product with pharmacological activities, dalpatein is a promising candidate for drug development with lesser adverse effects or toxicity with effective concentrations. Together, these results indicate that zebrafish are suitable model organisms to study the toxic effects.

**Table 27: Fish Embryo Toxicity studies- Lethal Endpoint**

| S.No | Dose | Coagulation | | | | Lack of somite formation | | | | Lack of detachment of tail | | | | Lack of Heart rate | | | | Death | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hrs | | 48 hrs | | 24 hrs | | 48 hrs | | 24 hrs | | 48 hrs | | 24 hrs | | 48 hrs | | (72 hrs) | |
| | | Dalpatein | DSEE | Dalpatein | DSEE | Dalpatein | DSEE | Dalpatein | DSEE | Dalpatein | DSEE | Dalpatein | DSEE | Dalpatein | DSEE | Dalpatein | DSEE | Dalpatein | DSEE |
| **1** | **Water** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **2** | **DMSO** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **3** | **1 µg/L** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **4** | **10 µg/L** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **5** | **100 µg/L** | - | 1 | - | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | - | - | 1 | 2 | 1 | 2 |
| **6** | **1 mg/lL** | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 3 | 2 | 3 | 3 | 3 | - | - | 3 | 4 | 3 | 4 |
| **7** | **10 mg/L** | 2 | 2 | 4 | 3 | 4 | 3 | 4 | 3 | 4 | 3 | 4 | 4 | - | - | 4 | 5 | 4 | 5 |

FIG9.3 PERCENTAGE LETHALITY OF DALPATEIN

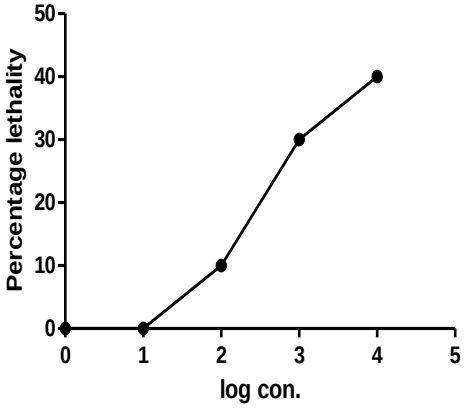
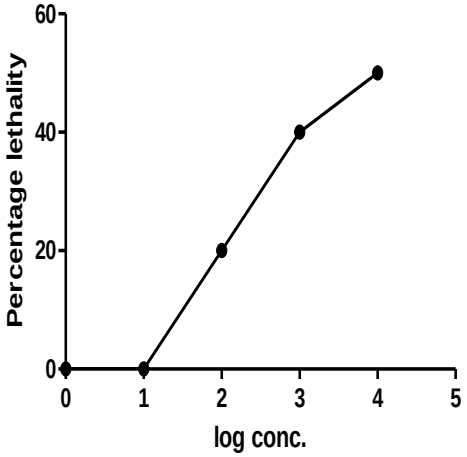



FIG 9.4 PERCENTAGE LETHALITY OF DSEE




**Anti obesity and lipid lowering effect** [229-241]**:**

Obesity is one of the major health threats of this century, which has an important impact on lifestyle-related diseases such as coronary heart disease, dyslipidemia, hypertension, glucose intolerance, diabetics, and some cancers. Several factors, such as lack of exercise, sedentary lifestyles and the consumption of energy rich diets are contributory to the etiology of obesity. There is an urgent need for safe, efficient therapeutics and the potential size of the market for anti-obesity drugs. The current status for the development of such drugs is still unsatisfactory .Some edible medicinal plants have been used as dietary supplements for body-weight management and control in many countries. Supplementation of the dalpatin from DSEE of *D.sissoo* have potent antioxidant potential could lower plasma lipid concentrations, Triglycerides, total cholesterol , SGOT and SGPT levels in zebra fish with High fat diet (HFD)-induced obesity.

**Table: 28 Serum profile and inhibitory activity of *Dalbergia sissoo* in zebrafish after 4 weeks of treatment**

| S.N | Description | Group I | Group II | Group III | Group IV | Group V | Group VI | Group VII |
|---|---|---|---|---|---|---|---|---|
| | | Normal | Control | Orlistat | 5 % DSEE | 10 % DSEE | 25µg Dalpatein | 50µg Dalpatein |
| 1 | **Weight (mg)/height (mm)** | 14.8 ± 4.3 | 16.25 ± 3.4[a] | 15.18 ± 3.3[b] | 16.24 ± 3.1[c] | 16.04 ± 3.1 [c] | 15.84 ± 3.9[d] | 15.21 ± 3.0 [d] |
| 2 | **Total cholesterol (mg/dL)** | 146 ± 2.5 | 490 ± 5 [a] | 201 ± 4.2 [b] | 394± 2.4 [c] | 326 ± 2.4 [c] | 296± 2.4 [d] | 224 ± 3.2 [d] |
| 3 | **Triacylglyceride (mg/dL)** | 113 ± 3.7 | 322 ± 4.1 [a] | 129 ± 5.7 [b] | 298 ± 3.9 [c] | 257 ± 4.5 [c] | 181 ± 3.8 [d] | 131 ± 3.5 [d] |
| 4 | **GOT (Karmen/mL)** | 70 ± 2.0 | 167 ± 3.6 [a] | 119 ± 4.8 [b] | 162 ± 3.8 [c] | 151 ± 3.7 [c] | 148 ± 3.2 [d] | 121 ± 4.7 [d] |
| 5 | **GPT (Karmen/mL)** | 10 ± 2.8 | 26 ± 3.7 [a] | 11 ± 3.9 [b] | 17 ± 4.2 [c] | 16 ± 4.5 [c] | 12 ± 4.4 [d] | 11 ± 2.7 [d] |

Values expressed as mean SEM *p<0.001

Data were expressed as mean ± SEM, n=10 per group.

ap<0.001 vs control group.

bp<0.001 vs orlistat group.

cp<0.001 vs Dalbergia sissoo treated group.

dp<0.001 vs Dalpatein treated group.

**Body weight and food intake**

As shown in Table, body weight gain of the HFD group zebra fish were considerably higher than the other groups. The weight gain of orlistat, dalpatein and ethanolic extract groups were almost normalized to the ND group. Feed intake of all experimental groups had no significantly different.

**Table: 29 Effects of drugs on body weights in zebra fish fed high-fat diet**

| Body weight | Parameters | | |
|---|---|---|---|
| | Initial body weight (mg) | Final body weight(mg) | Body weight gain (mg/study) |
| **Normal** | 724±1.87 | 728±1.92 | 4 |
| **High fat diet** | 638±2.91 | 674±2.67 | 36 |
| **Orlistat** | 754±1.52 | 760±1.09 | 6 |
| **0.5 mg DSEE** | 752±1.77 | 774±1.65 | 22 |
| **1 mg DSEE** | 711±0.98 | 728±1.27 | 17 |
| **25 µg dalpatin** | 684±1.67 | 694±1.06 | 10 |
| **50 µg dalpatin** | 697±1.95 | 705±1.87 | 8 |

*Values expressed as mean± SEM

*D. sissoo* has been traditionally used as food and used in folk remedies and some of its pharmacological actions have been partly confirmed by modern science. Although a high intake of polyphenols and flavonoids significantly reduced the risk of obesity and act as lipid lowering agent.

The present study revealed that body weight gain of the HFD group zebrafish were not significantly higher than in the normal group, and the weight gain of *Dalbergia sissoo* ethanolic extract, dalpatein and orlistat groups were normalized to the ND group. There were no abnormalities in growth performance; the relative weight of the liver had no significantly higher in the HFD group compared with other groups. Interestingly, feed intake of all experimental groups had no significantly different [244-246].

High-fat diets significantly increase the Total cholesterol and triglycerides levels in the serum and liver as compared with the normal control diet in zebrafish. The effects of polyphenols or flavonoids on lipid profile are very relevant to metabolic disorders. The anti-obesity function improved lipid profile by lowering serum total cholesterol, TG compared with the HFD group.

Intestinal lipase inhibition using orlistat has been widely used in the pharmacotherapy of morbid obesity. The present study showed that dalpatein and DSEE were comparable to orlistat in anti-obesity and improving lipid profile aspects of zebrafish fed high-fat diet.

HC group had a 3.0-fold increase in serum cholesterol and TG, respectively, compared to normal group following HC feeding With the increase in the lipid profile, serum GOT and GPT levels were also 2.7-fold increased compared to normal group (Table 29). These results suggest that dalpatein and ethanolic extract had potent and anti-obesity activity with reduction of hepatic inflammation parameters.

**Effectiveness of dalpatein and ethanolic extract in inhibition of pancreatic lipase:**

Orlistat is one of the drugs that usually used to treat obesity and shows the activity as an inhibitor of pancreatic lipase [8]. In the body, orlistat prevents the absorption of dietary fats and it works by inhibiting pancreatic lipase in the breakdown of triglycerides into free fatty acid and monoglyceride. When pancreatic lipase activity is inhibited by orlistat, triglycerides from food cannot be hydrolyzed into free fatty acids, but immediately expelled from the body through the faeces.

Orlistat has a unique structure that is able to bind the active side of the lipase and thus inhibit the activity. Orlistat will compete with substrates of triglyceride to bind to the active side of the lipase. Lipase enzyme that has been bonded with orlistat was not able to bind to triglyceride, and vice versa. Because most of the enzyme has been bound to orlistat, most of the enzymes cannot catalyze the hydrolysis of TG. Thus, the amount of free fatty acids as products of hydrolysis reactions was decreased.

**DOCKING STUDIES OF LIGANDS WITH PL PROTEINS:**

Attempts were made to understand the hypothesis, mechanism of action of the selected herbal drug in controlling the pancreatic lipase (PL) through docking tools. Identified the drug molecule through metabolomic approach was subjected to docking studies. The anti obesity potentials of the drug molecule will be determined based on the hydrogen bond formation and energy release.

Hence to prove the pharmacodynamic of dalpatein, the computational simulation studies were done with the dalpatein with PL-Colipase complex to propose the above physiological response.

Molecular docking was performed on dalpatein to understand the affinity and mode of binding with the PL enzyme active site residues. Docking analysis showed a high binding potential towards the active site of PL with total docking score of −6.54 kcal/mol and the results are listed in the table 30.

Table: 30 Results of molecular docking of dalpatein to PL-Colipase complex

| Est. Free Energy of Binding | Est. Inhibition Constant, Ki | vdW + Hbond + desolv Energy | Electrostatic Energy | Total IntermolecEnergy | Frequency | Interact Surface |
|---|---|---|---|---|---|---|
| -6.54 kcal/mol | 15.97 uM | -6.92 kcal/mol | -0.10 kcal/mol | -7.01 kcal/mol | 100% | 492.118 |

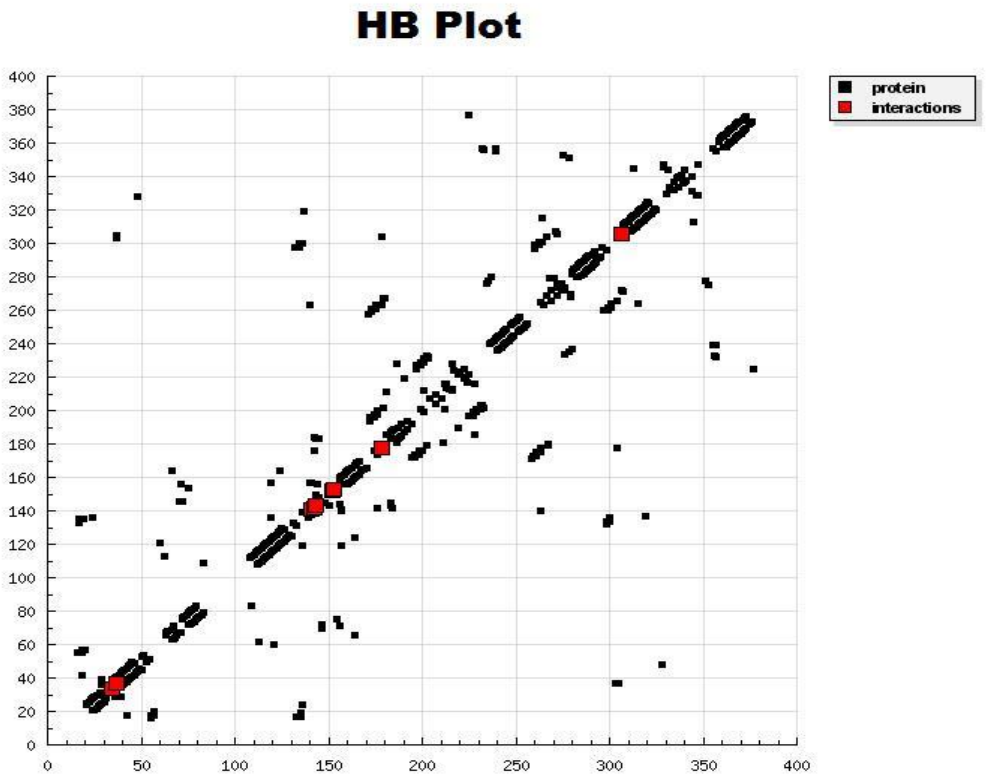



Fig: 10. 4 HB plot showing interaction of proteins

Interaction protein for dalpatein from the HB plot 34: ILE, 37: ARG, 141: TRP, 142: HIS, 143: HIS, 152: PHE, 153: CYS, 178: ASP, 306: TYR.

The docking score of dalpatein along with the hydrogen bonding interaction residues are illustrated in table 31.

Table: 31 Interaction Table

| hydrogen bonds | Polar | hydrophobic | cation-pi | other |
|---|---|---|---|---|
| H1 () [*3.42*] – TYR306 (*CE2, OH*) | O2 () [*3.86*] – ARG37 (*NH1*) | C3 () [*3.37*] – TRP141 (*CE3, CZ3*) | H2 () [*3.74*] – TRP141 (*CZ3*) | H1 () [*3.16*] – ILE34 (*CB, CD1, CG1, CG2*) |
| | H2 () [*2.98*] – TYR306 (*OH*) | C1 () [*3.63*] – TRP141 (*CH2, CZ3*) | H1 () [*3.11*] – TRP141 (*CH2, CZ3*) | O1 () [*3.72*] – ILE34 (*CG1, CG2*) |
| | O1 () [*3.64*] – TYR306 (*OH*) | C9 () [*3.58*] – HIS142 (*CD2*) | H2 () [*3.23*] – PHE152 (*CB, CD2, CG*) | O2 () [*3.72*] – ILE34 (*CD1, CG2*) |
| | | C4 () [*3.79*] – HIS143 (*CD2*) | | H1 () [*3.47*] – TRP141 (*CH2, CZ3*) |
| | | C5 () [*3.55*] – HIS143 (*CD2*) | | O2 () [*3.77*] – TRP141 (*CH2*) |
| | | C4 () [*3.20*] – CYS153 (*SG*) | | O1 () [*3.75*] – TRP141 (*CH2*) |
| | | C9 () [*3.10*] – CYS153 (*SG*) | | C7 () [*3.69*] – HIS142 (*NE2*) |
| | | | | C9 () [*3.83*] – HIS142 (*NE2*) |
| | | | | C5 () [*3.63*] – HIS143 (*NE2*) |
| | | | | C7 () [*3.31*] – ASP178 (*OD2*) |
| | | | | O1 () [*3.61*] – TYR306 (*CE2*) |

In human PL, N-terminal domain residues Ser152, Asp176, and His263 form the catalytic trial while C-terminal domain binds to co-lipase, the cofactor required for the activity. To achieve an active conformation, the open lid structure of the PL requires the interactions of Arg257 (Arg256 in PDB: 1LPB) and Asp258 (Asp257 in PDB: 1LPB) with the core residues. Any disruption of the interactions with these residues prevents the lid from attaining an optimal conformation.

From the docking pose of ligand as shown in Fig. 10.5 within the PL enzyme active site, it is clearly observed that dalpatein formed strong hydrogen bonding interactions with the active site of amino acid residues of the enzyme (Table 31). The compounds binding to these catalytic and other nearby residues are expected to play an important role in PL inhibition [252 & 253] supporting the stronger PL inhibitory activity of the dalpatein in our in vitro assay (Table 31). Interestingly, the trend of variation of the PL inhibitory activity ($IC_{50}$) and docking score of these compounds was excellent, and highly significant correlation coefficient of 0.62 was obtained. These results clearly suggest that these dalpatein have definite effect on lipid metabolism and their effects may be due to inhibition of PL reducing the intestinal absorption of dietary fats.

# CHAPTER X

# CONCLUSION

Plant possesses an estimated value of 200,000 metabolites with different niche properties to increase our curiosity. Nature evolved this metabolite by the million years of hard work and screening so they are the fittest candidate on Darwin principles. (That's why most of the drugs are simply natural compounds or their Analogs.) There are many unknown plants with high medicinal value still have not been recognised their importance. They have not been brought to the light of scientific world. The pharmacopoeial standards in Ayurvedic Pharmacopoeia of India are not adequate enough to ensure the quality of plant materials since the materials received in the manufacturing premises are not in a condition that effective microscopic examination can be done.

This dissertation covers pharmacognostical, phytochemical and pharmacological studies with a modern metabolomic approach on the commonly available medicinal plant *Dalbergia sissoo* belonging to the family Fabaceae.

The purpose is to link the traditional concepts like the role of herbal drugs, herbal products and certain phytochemicals for potential phytomedicine using modern scientific approaches. The objective is to examine the impact of enriched natural herbals on obesity which is one of the primary causes for the associated diseases like CVD risk, atherogenesis, diabetes, antioxidant potential, cancer, neurocognitive function and eye disease.

Pharmacognostical parameters have been determined on the leaves inorder to substantiate and identify the plant for future work. This study establishes not only

pharmacognostic and characterizations of leaves but also microscopic and fluorescences. These characteristics can be used further as identification and authentication parameters of the leaves.

Induction of callus using plant tissue culture technique provides the detailed procedures including explant preparation, inoculation of explant and callus induction are described. Explant was inoculated in MS basal medium and MS basal medium supplemented with different concentrations of BAP and NAA. In this 3.0 mg/L BAP + 0.5 mg/L NAA combination produced highest fresh weight of callus per culture and it was 10.3 g.

Highest extractive value from leaves (28.74 %) and callus (1.4%)of *D.sissoo* was obtained using 70 % alcohol. Preliminary phytochemical screening on the leaves of *D.sissoo a*nd callus of *D.sissoo* confirms the presence of flavanoids, phenols, tannins, sterols, glycosides, carbohydrates and saponins.Thequantification of total phenol, flavonoid and tannin was carried out for ethanolic extract of leaves of *Dalbergia sissoo* and ethanolic extract of calli of *Dalbergia sissoo* were found to be **88.98±3.0 mg/g and 125.12±1.8 mg/g, 47.64±1.8 mg/g and 82.53±0.5 mg/g and 281.01 mg/g and 413.09 mg/g** respectively.It confirms the significant concentration of these phytoconstituents in the ethanolic extract of calli of *Dalbergia sissoo* than the ethanolic extract of leaves of *Dalbergia sissoo* which satisfy our aim and standing proof for the modern approachfor the production and analysis of secondary metabolites from the cultured callus of *D. sisoo* using for the greater physiological functions so their significance in pharmacology must be very high. Hence our results prove that the modern tissue culture technique is rapid, inexpensive method to isolate high concentration and disease resistance secondary metabolite for effective treatment of ailments for the herbal drug development in near future.

Isolation of active compound from the extract of leaves of *D.sissoo* was done using column chromatography and structure was elucidated by spectral methods of metabolomics studies. An isoflavanoid with molecular weight 341.17with IUPAC name 7-hydroxy-6-methoxy-3-(6-methoxy-1,3-benzodioxol-5-yl)chromen-4-oneand identified as dalpatein (Pubchem -CID 25203443).

Pharmacological screening confirms the ethanolic extract of leaves, ethanolic extract of callusand isolated compound Dalpatein as potent radical scavenger from DPPH assay, hydrogen peroxide assay and total anti oxidant using phosphomolybdenum method. It was evident from the phytochemical studies of this plant, that substantial amount of phenols, tannins and flavanoids were present in these extracts which exhibited significant *invitro* antioxidant activity as ascorbic acid.

Various functional components, such as flavonoids and polyphenols in *D.sissoo*, could play important roles in altering body fat and regulating lipid metabolism. This claim was further confirmed by in vitro lipase enzyme inhibition in chicken pancreas by 55% ($IC_{50}$- 2.39 mg/ml) there by matches with marketed orlistat by 68% ($IC_{50}$-2.04 mg/ml) which is used as standard.

*In vivo* anti obesity therapeutic potential was studied after a detailed acute toxicity studies in zebrafish embryos with $LD_{50}$ (DSEE-10mg and dalpatein 100mg). A significant anti obesity activity was observed at 1mg of ethanolic extract of leaves ($p<0.001$), 50µg of Dalpatein ($p<0.001$) against high fat induced in zebra fish as compared to orlistat.

The pharmacodynamic properties of dalpatein were elucidated from the computational simulation studies with PL-Colipase complex of humans to propose the above physiological responses for the activity.

Molecular docking showed the affinity and mode of binding with the PL enzyme active site residues by dalpatein with total docking score of −6.54 kcal/mol with OH of TYR306 having inhibition constant ($k_i$) of 15.97µM.

Orlistat is one of the drugs that is usually used to treat obesity and shows the activity as an inhibitor of pancreatic lipase. Hence our an integrative *in vivo-in vitro-in silico* approach of dalpatein will prove to be a novel herbal drug which prevents the absorption of dietary fats and it works by inhibiting pancreatic lipase in the breakdown of triglycerides into free fatty acid and monoglyceride. When pancreatic lipase activity is inhibited by our potent phytomedicine, triglycerides from food cannot be hydrolyzed into free fatty acids, but immediately be expelled from the body through the faeces.

Thus the present study may have important implications because it is the first report that *D. sissoo* leaf extract has anti-obesity and improving lipid profile effects using the metabolomic studies for a potent phytomedicine in near future. It may also serve as a lead molecule to synthesise various semisynthetic drugs to treat life threatening disease OBESITY.

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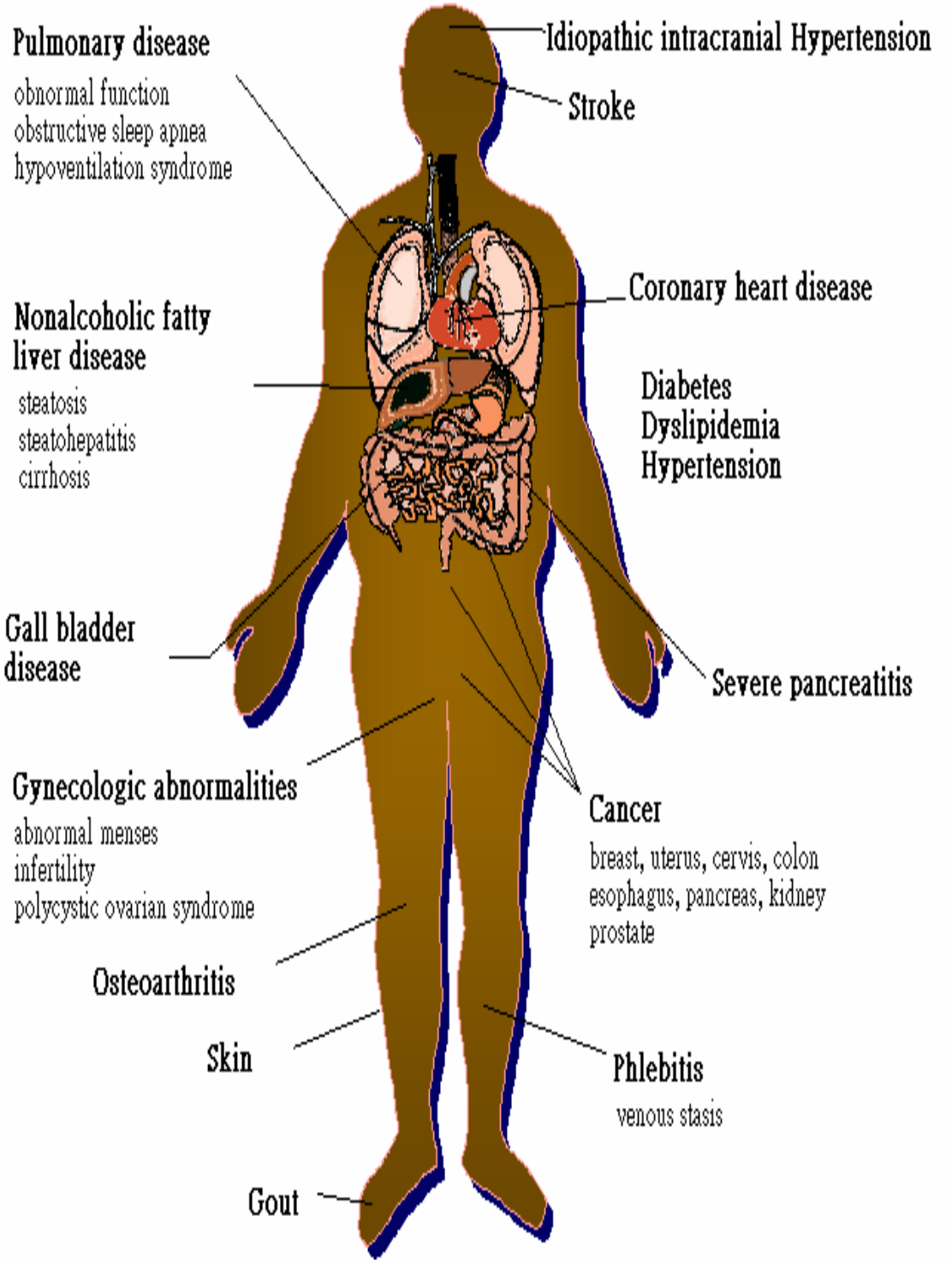
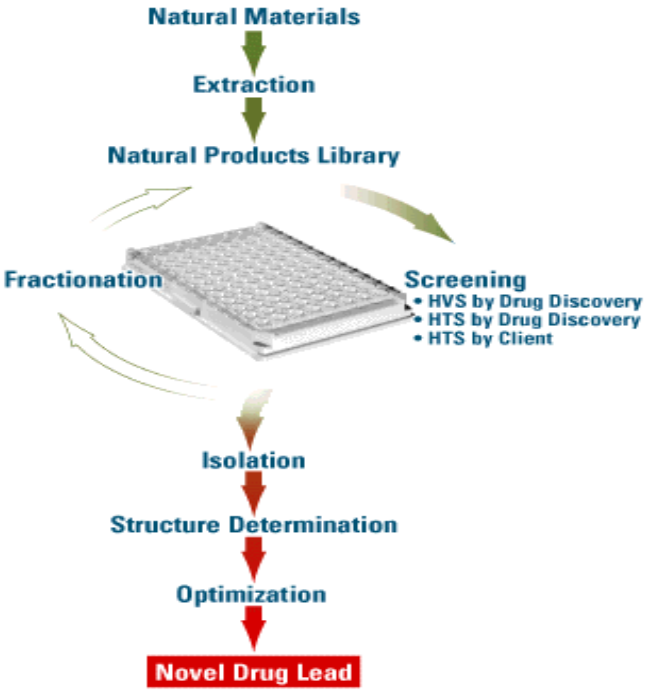
## FIG 1.1 MEDICAL COMPLICATIONS OF OBESITY



## FIG 1.2 NOVEL DRUG FROM NATURAL PRODUCT




## FIG 1.3 NATURAL PRODUCTS ROLE IN OBESITY CONTROL

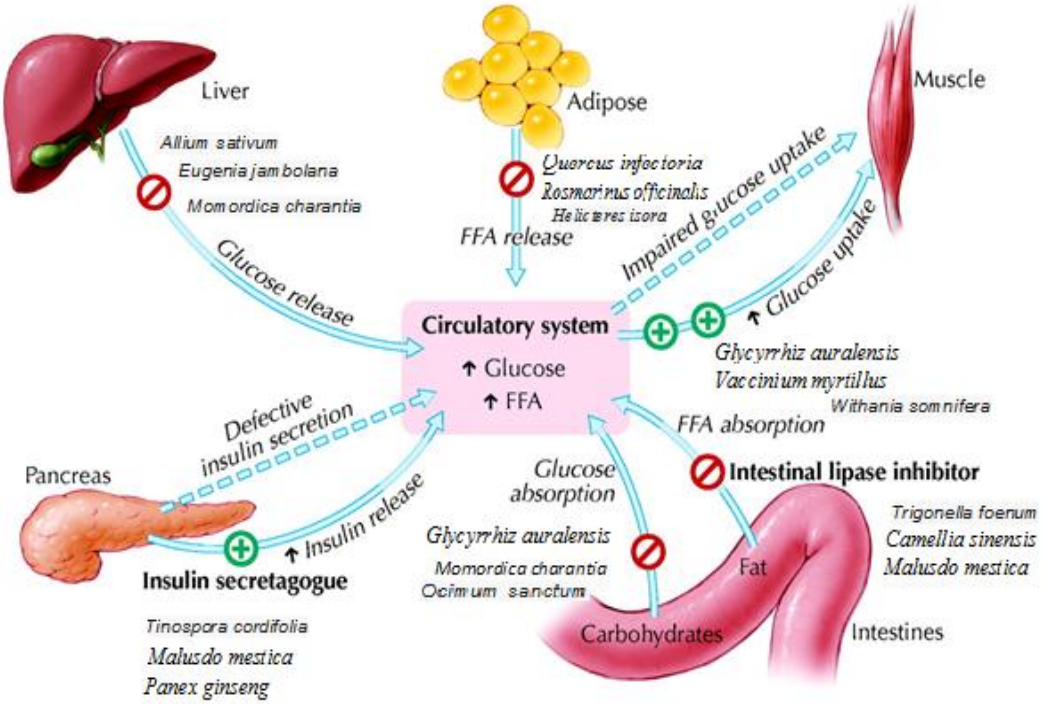


**FIG 1.4 SCHEMATIC WORKFLOW OF A METABOLOMICS APPROACH FROM TISSUE HARVEST THROUGH TO DATA INTERPRETATION USING COMPLEMENTARY ANALYTICAL INSTRUMENTATION FOR GREATER COMPREHENSIVENESS OF METABOLITE DETECTION AND QUANTIFICATION**

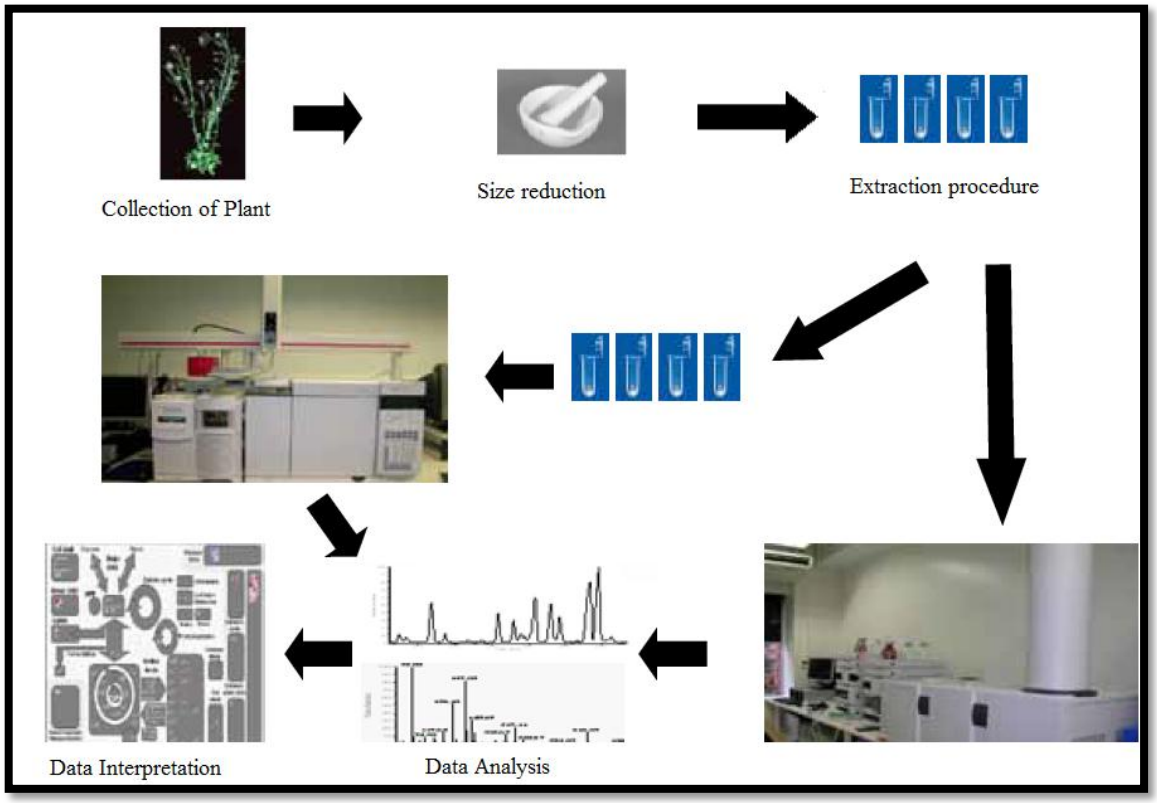


FIG 2.1 HABIT AND HABITAT OF *Dalbergia sissoo* Roxb.

FIG 2.2 DIAGRAMETIC REPRESENTATION OF *Dalbergia sissoo* Roxb.

# FIG 2.3 DORSAL VIEW AND VENTRAL VIEW OF LEAF


FIG 2.1 HABIT AND HABITAT OF *Dalbergia sissoo* Roxb.

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## FIG 3.1 T.S OF MIDDLE PART OF PETIOLE


## FIG 3.2 T.S. OF PETIOLE- A PORTION ENLARGED

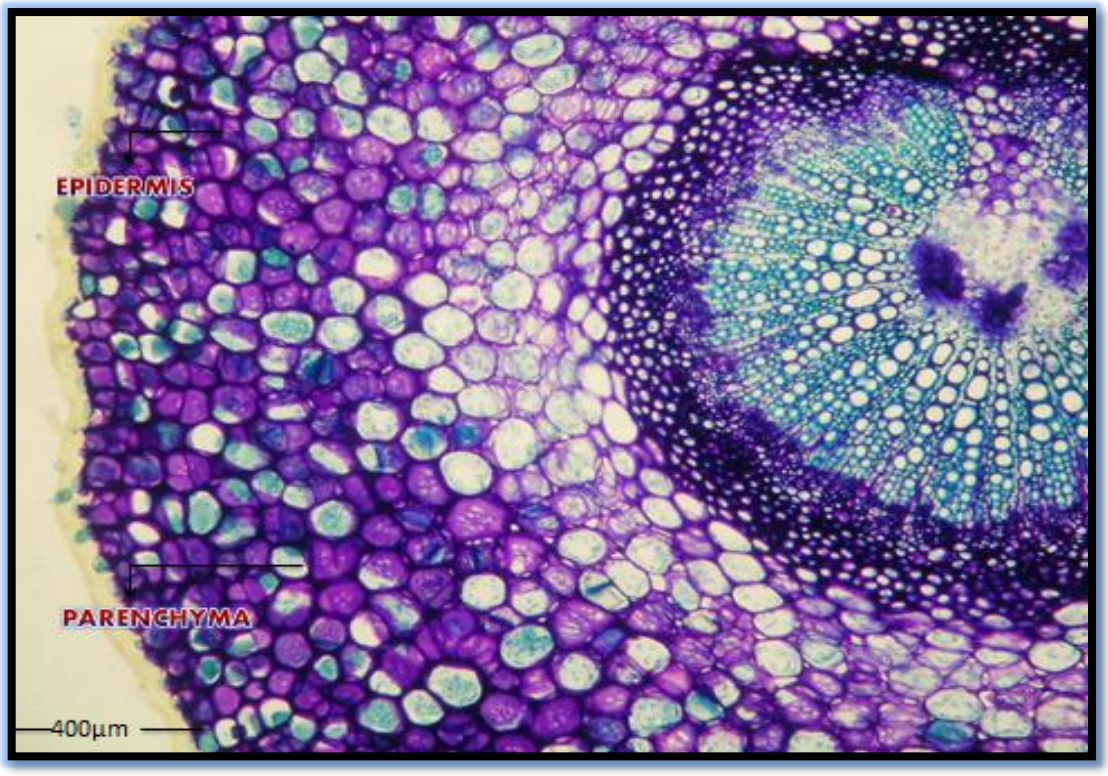


FIG 3.3 T.S. OF PETIOLE - SHOWING CORTICAL CELLS

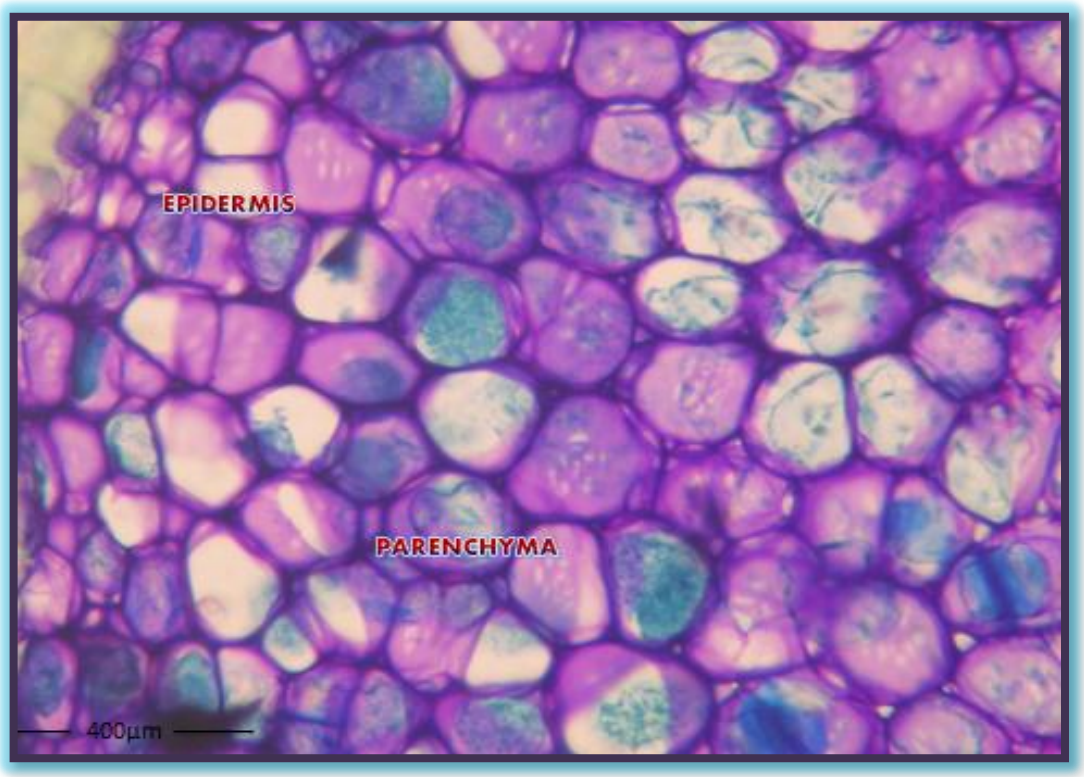
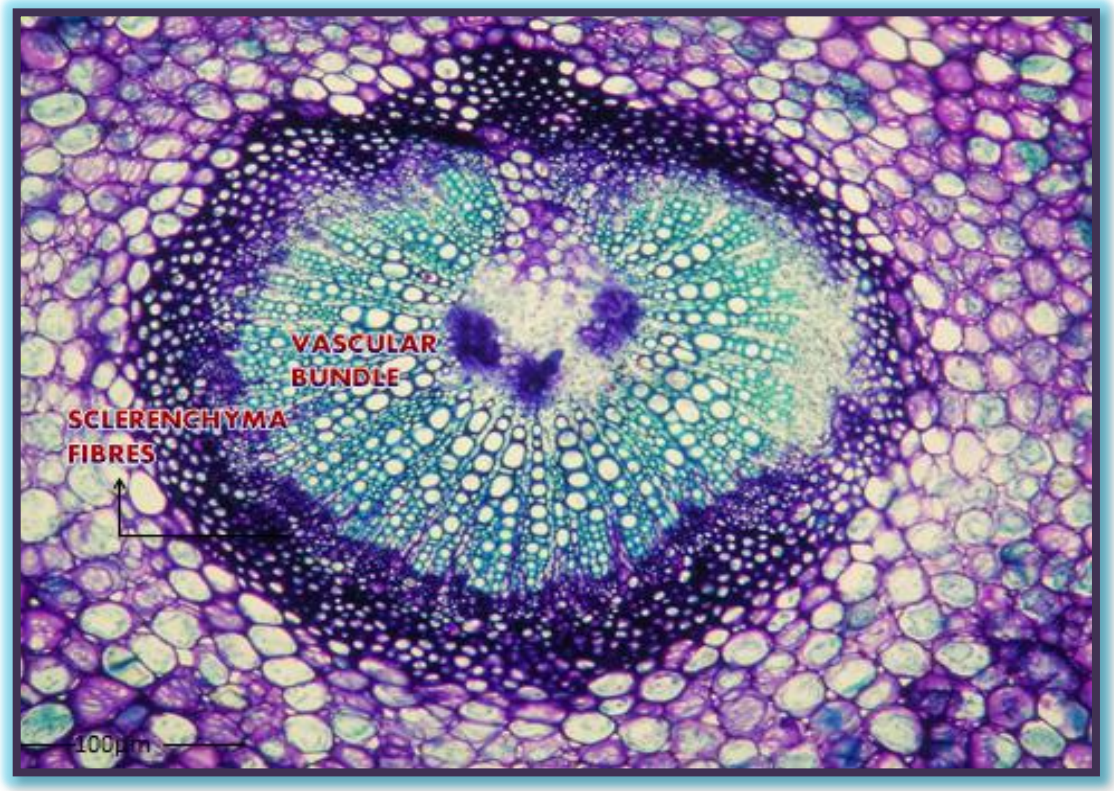
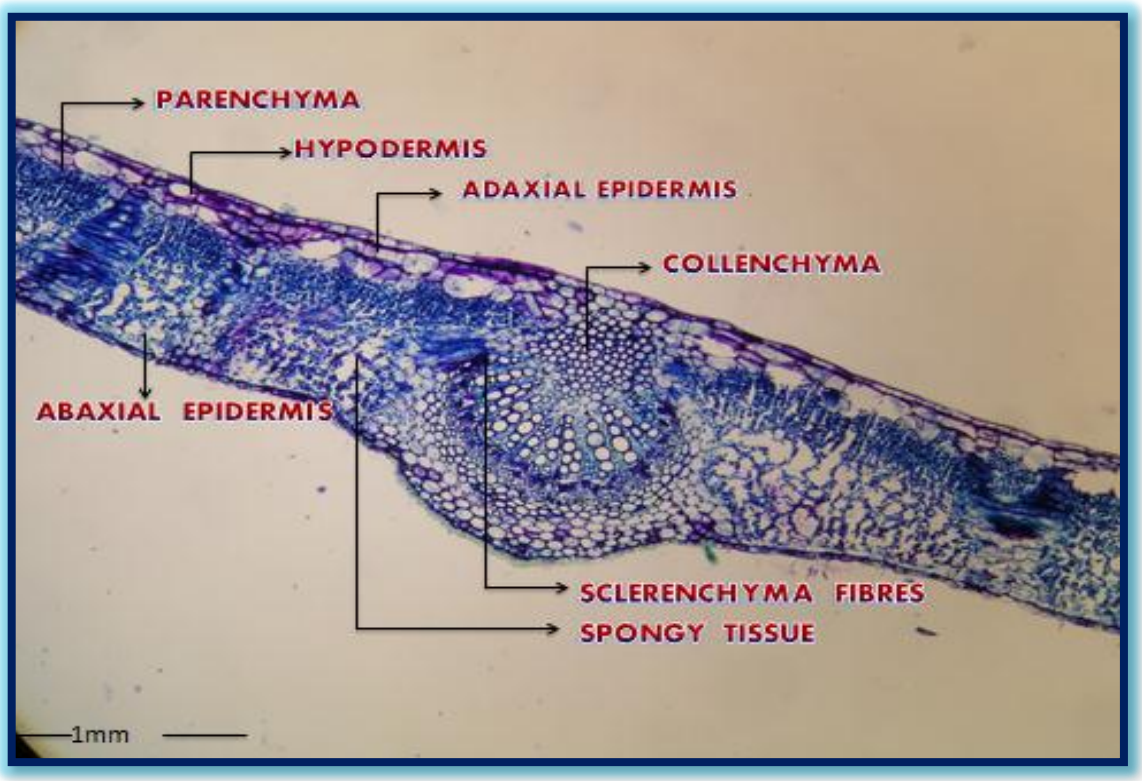



FIG 3.4 T.S. OF PETIOLE – VASCULAR BUNDLE ENLARGED



## FIG 3.5 T.S OF LEAF THROUGH MIDRIB




## FIG 3.6 T.S OF LAMINA ENLARGED

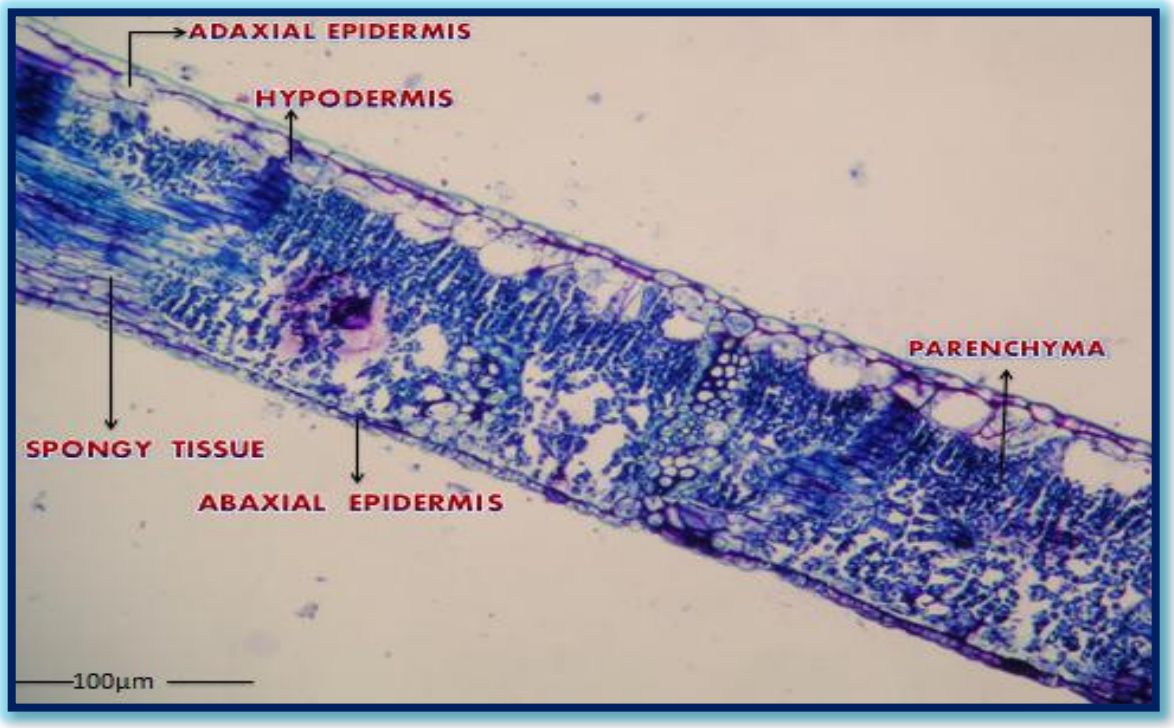
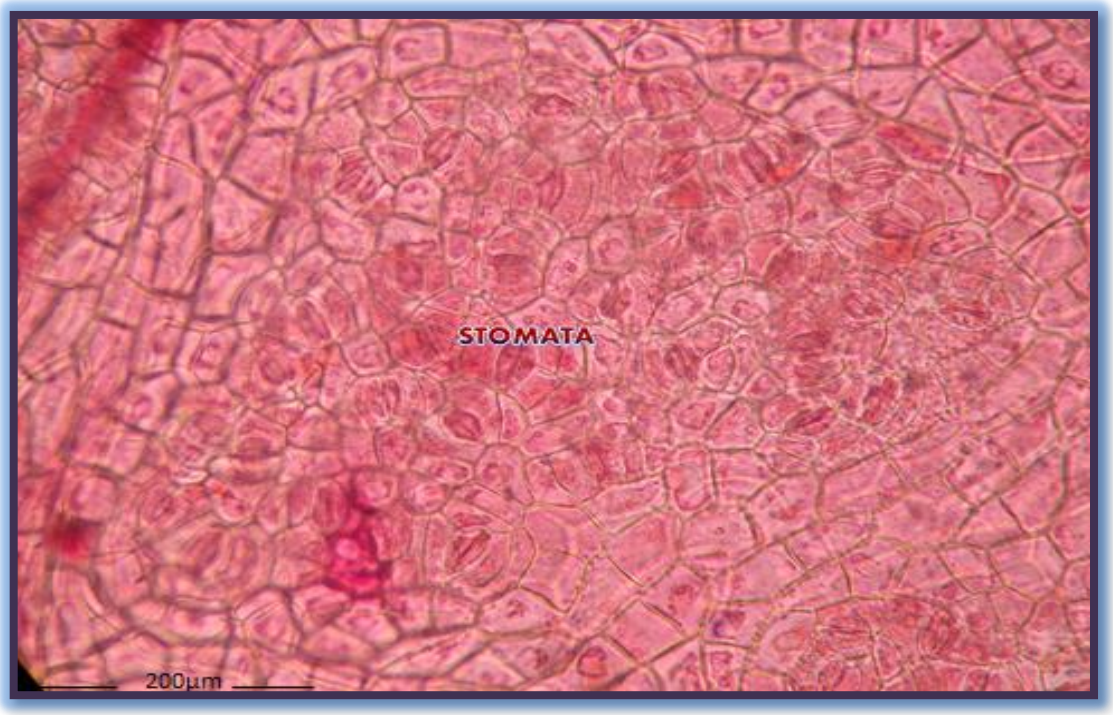


FIG 3.7 ADAXIAL FOLIAR EPIDERMIS SHOWING TRICHOMES


FIG 3.8 SURFACE VIEW OF STOMATA



## FIG 3.9 STOMATA – ENLARGED


## FIG 3.10 PARADERMAL SECTION SHOWING VENATION OF THE LAMINA

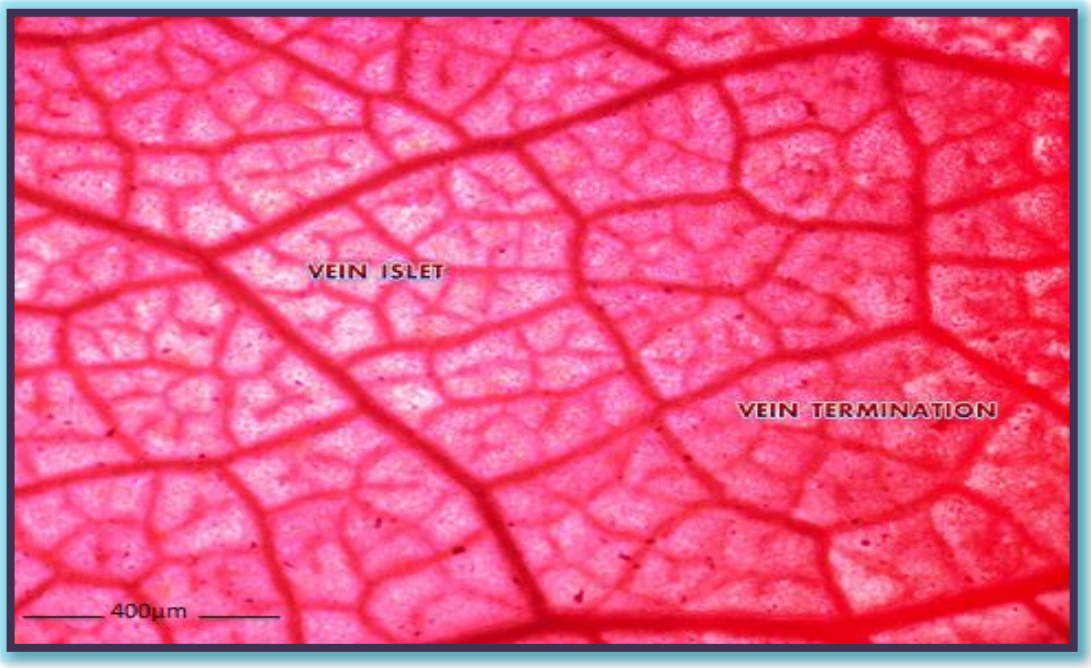


FIG 3.11 TRICHOMES

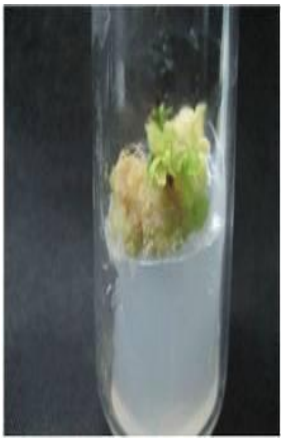

FIG 3.12 TRICHOME – ENLARGED

FIG 4.1 EXPLANT IS INOCULATED IN BASAL MEDIUM

FIG 4.2 CALLUS INDUCTION IN BM + BAP 3 MG/ L + NAA 0.5 MG / L ON 18$^{TH}$ DAY

FIG 4.3 CALLUS FORMATION AFTER 5 WEEKS IN BM + BAP 3 MG/ L + NAA 0.5 MG / L

FIG 4.4 CALLUS FORMATION IN BM + BAP 3 MG/ L + NAA 0.5 MG / L AFTER 3 MONTHS



# THIN LAYER CHROMATOGRAPHY

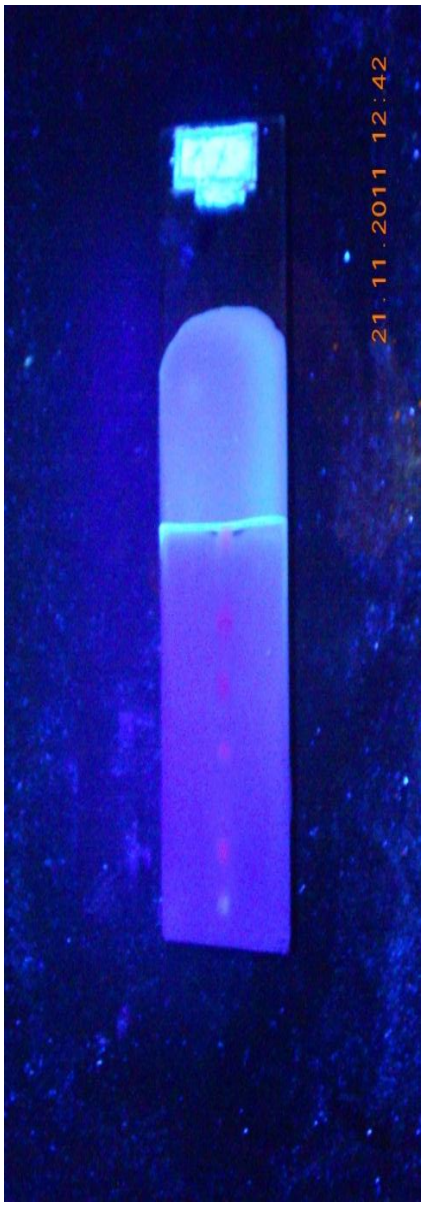



FIG: 5.1 TLC OF DSEE

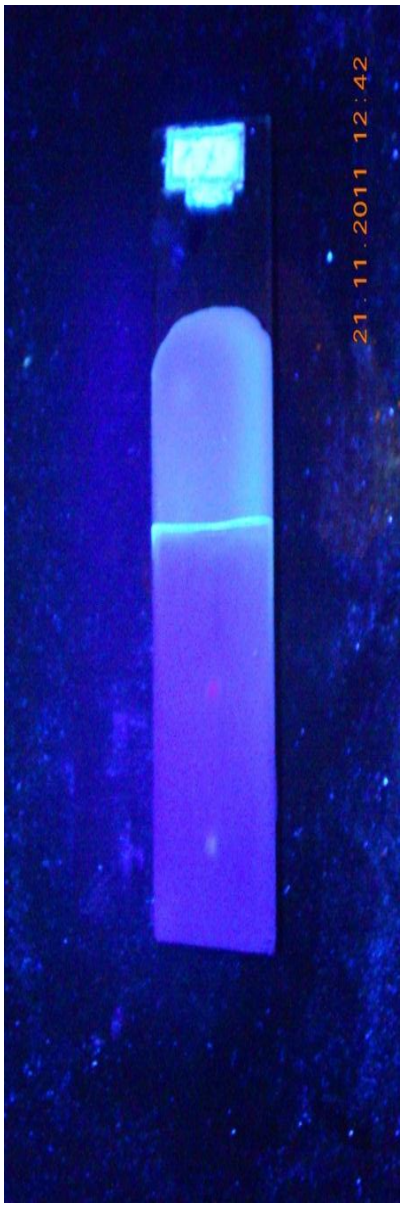



FIG: 5.2 TLC OF DSIF

## Fig: 5.3 UV DETERMINATION OF ISOLATED COMPOUND FROM *D.sissoo*

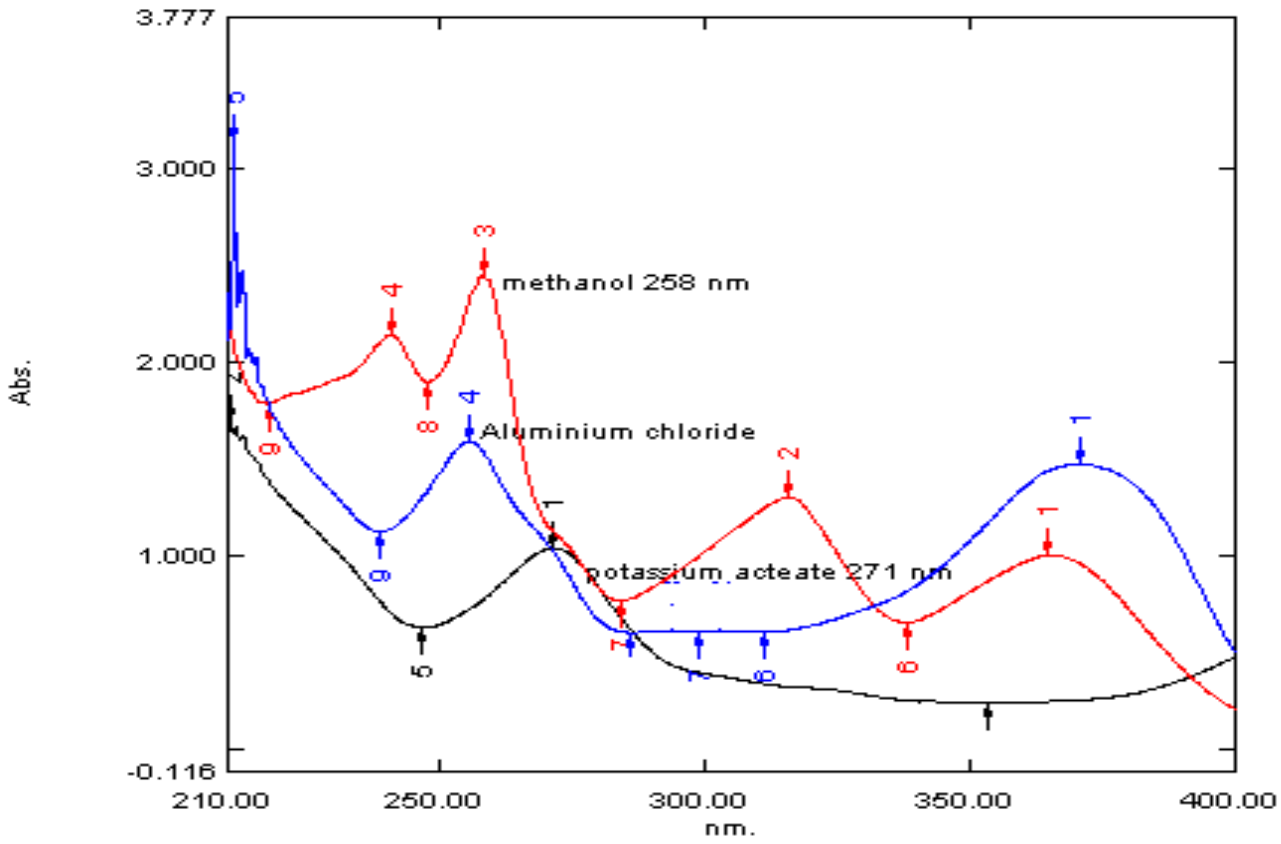


FIG 7.2 DERTERMINATION OF SCAVENGING ACTIVITY OF *D.sissoo* AGAINST HYDROGEN PEROXIDE

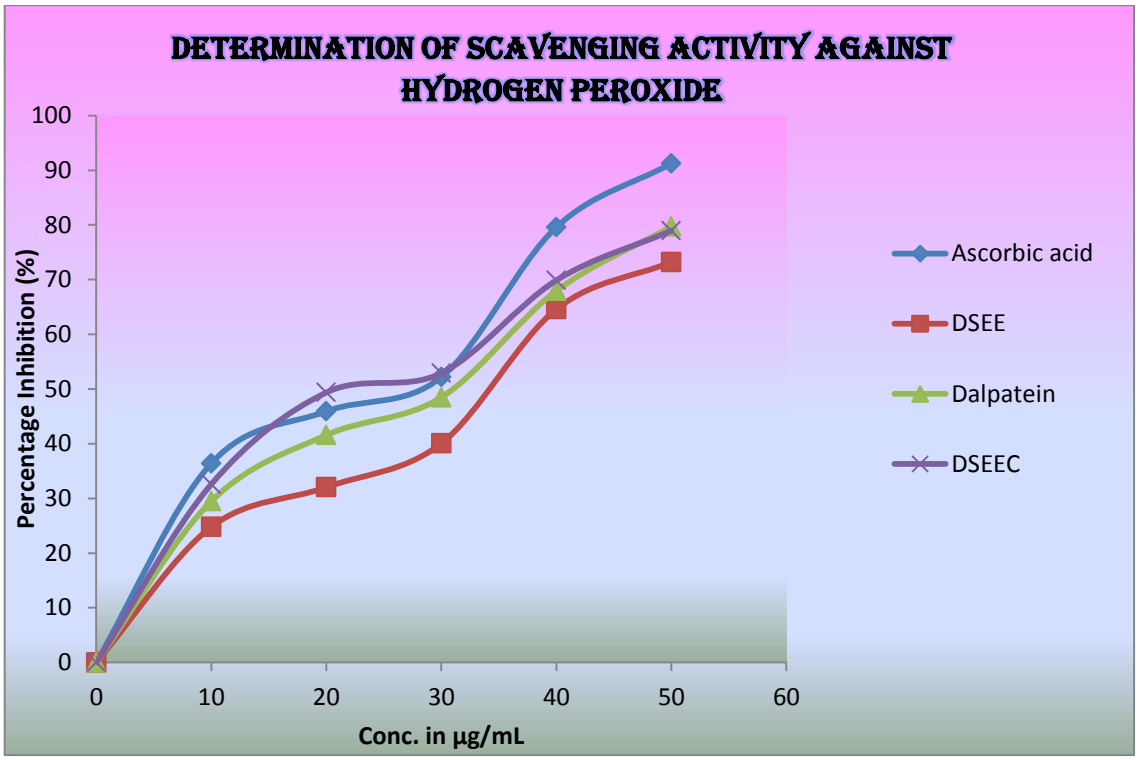
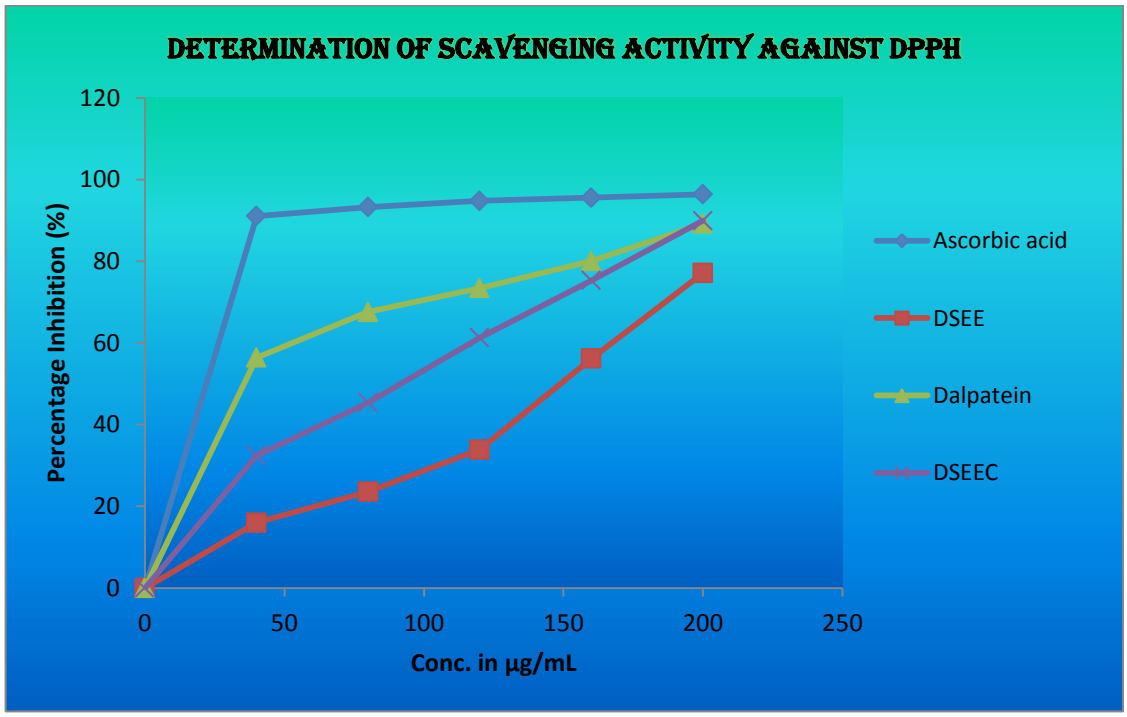
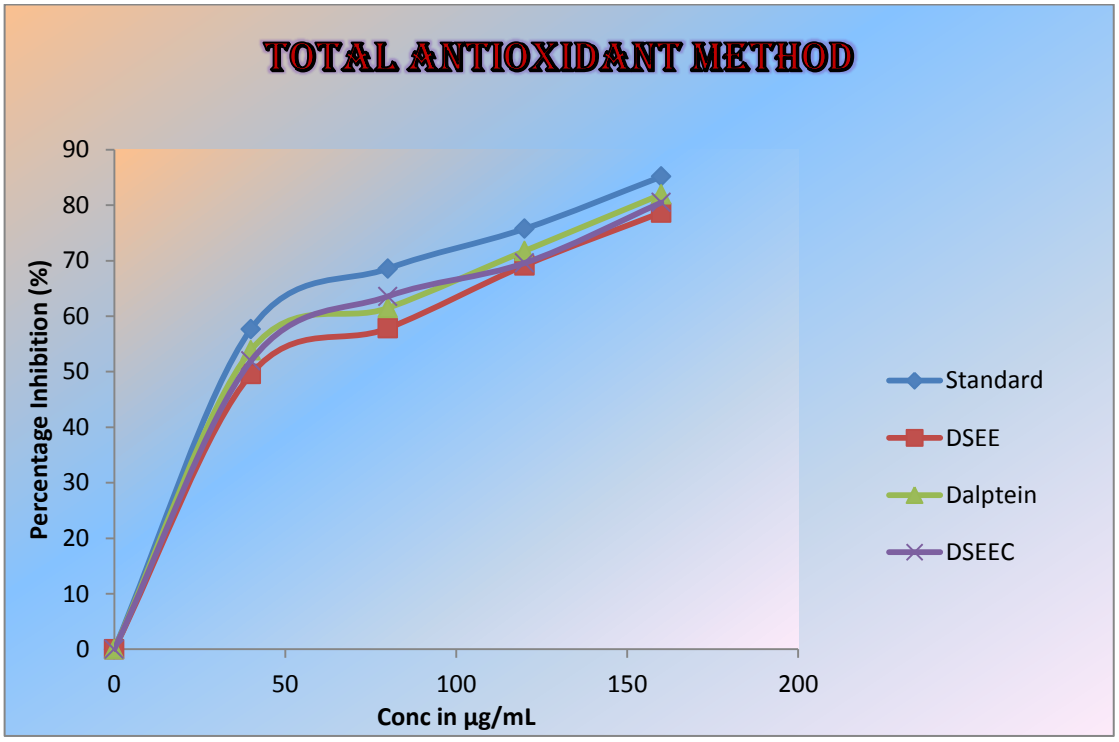


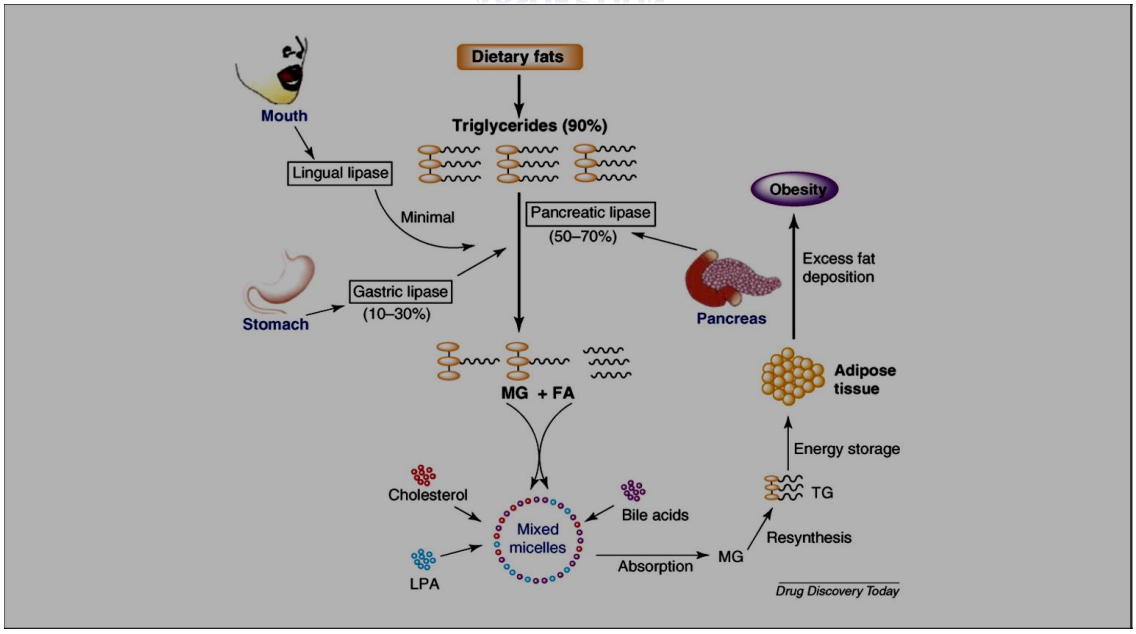

FIG 7.3 DERTERMINATION OF SCAVENGING ACTIVITY OF *D.sissoo* AGAINST DPPH



## FIG 7.4 TOTAL ANTI OXIDANT METHOD OF *D.sissoo*

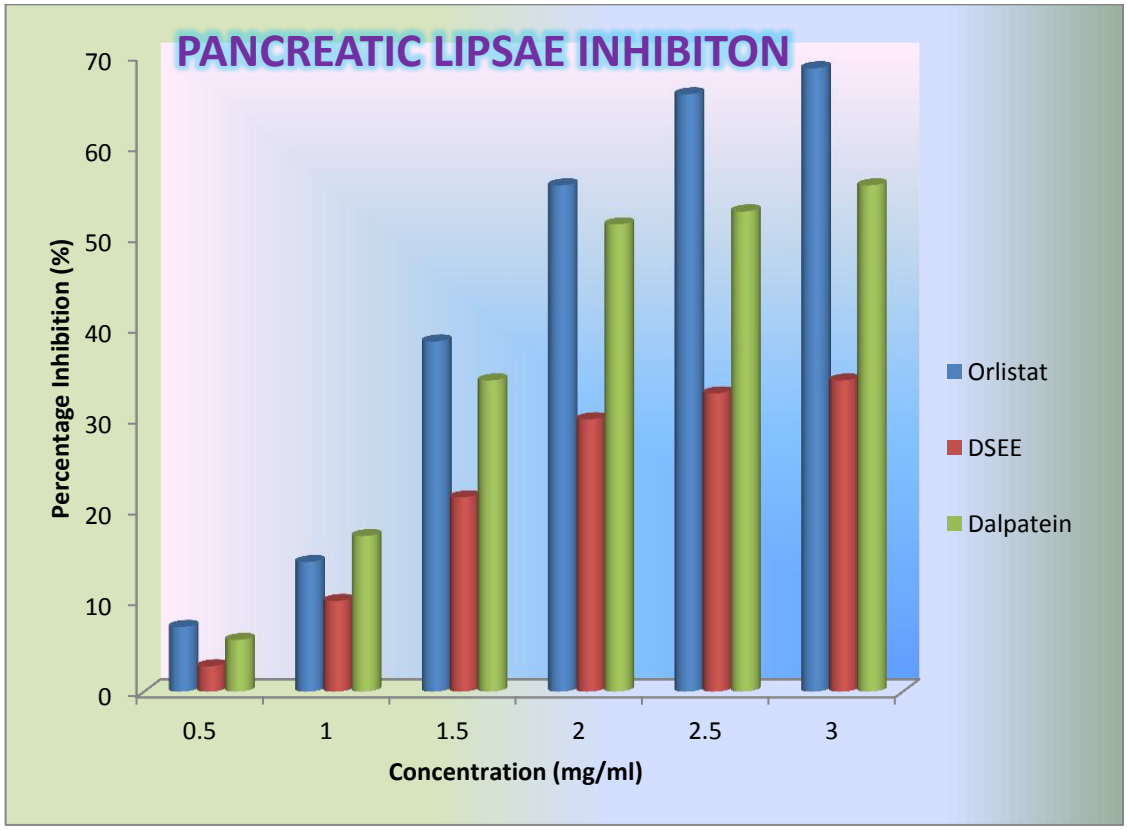


## FIG 8.1 PHYSIOLOGICAL ROLE OF PANCREATIC LIPASE IN LIPID ABSORPTION



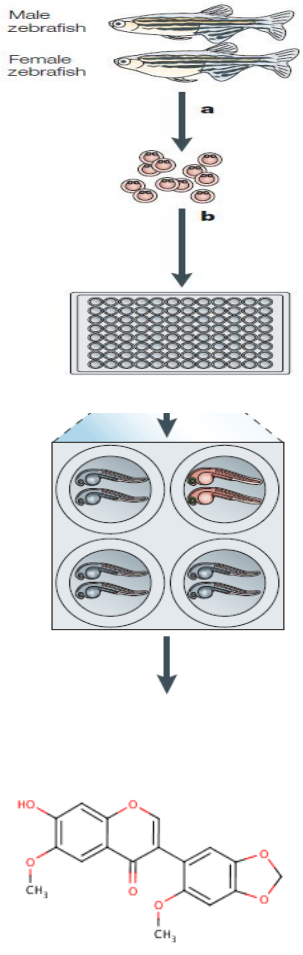

Dietary fats are mainly (90%) comprised mixed triglycerides (TGs) and are required to be hydrolyzed for their absorption. Of the various lipases, PL is the principle lipolytic enzyme accounting for the hydrolysis of 50–70% of dietary fats to their respective fatty acids (FA) and monoglycerides (MGs). The MGs and free FAs, released by lipid hydrolysis, form mixed micelles with bile salts, cholesterol and lysophosphatidic acid (LPA) and are absorbed into enterocytes where re synthesis of TGs takes place. TGs are stored in adipocytes as their main energy source.

## FIG 8.2 PERCENTAGE INHIBITION OF PANCREATI LIPASE

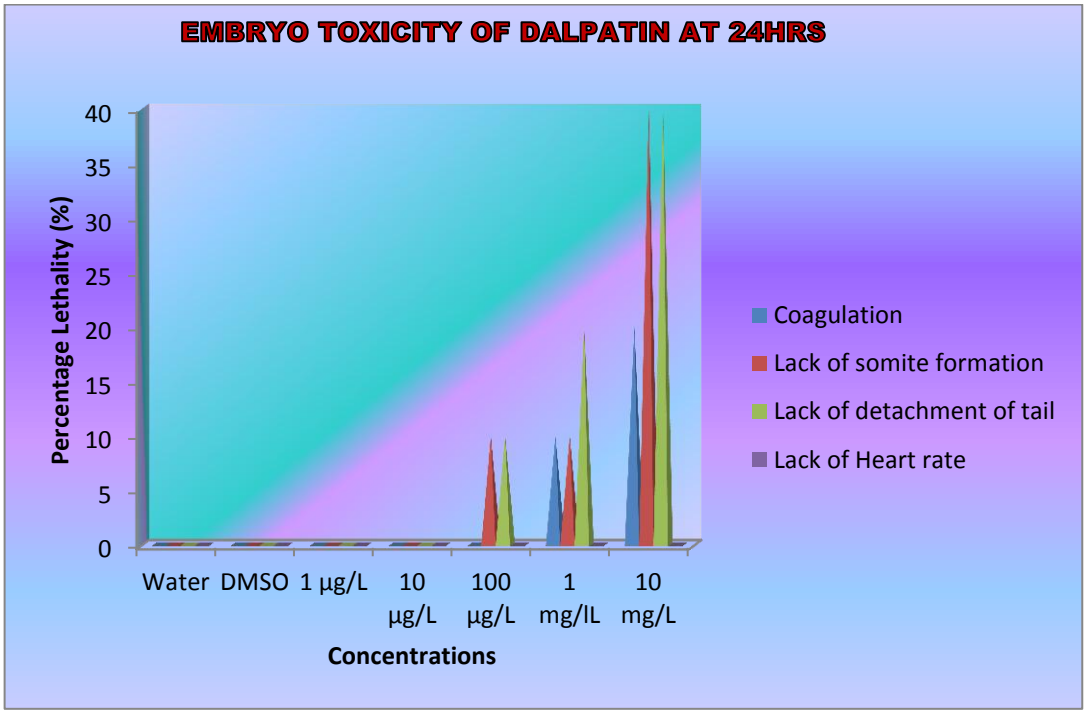


## FIG 9.1GENERAL FEATURES OF ZEBRAFISH THAT MAKE THEM EXCELLENT LABORATORY ANIMAL MODEL

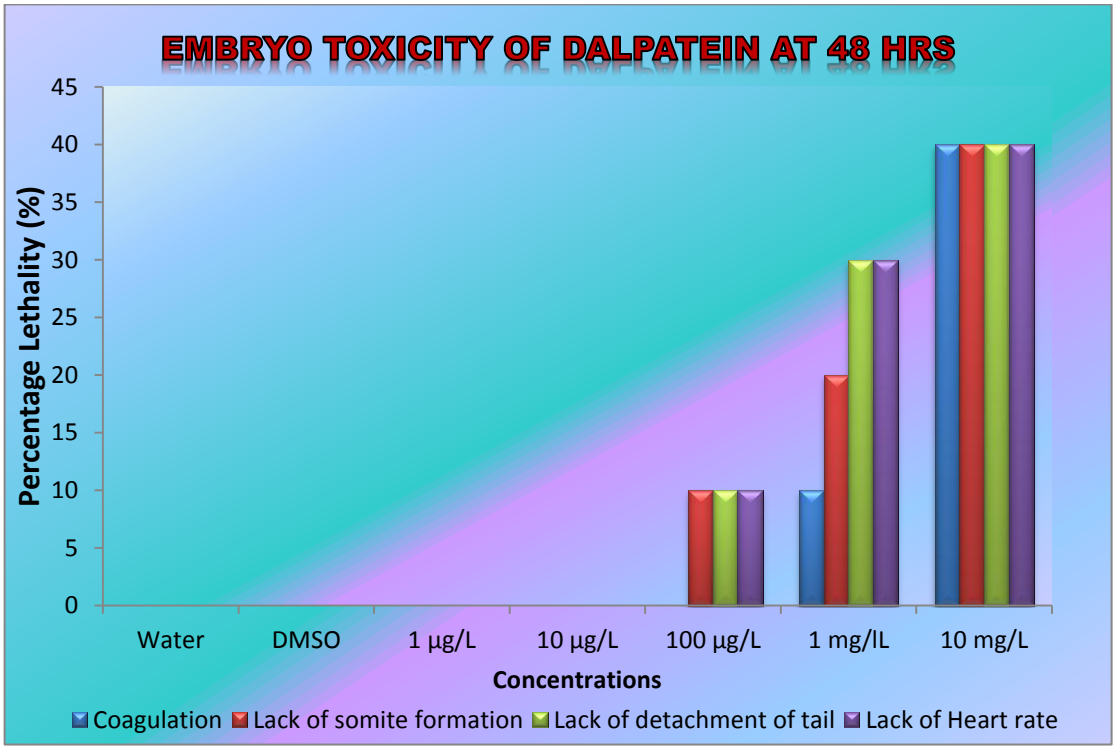

## FIG 9.2 ZEBRAFISH SMALL MOLECULE SCREENS



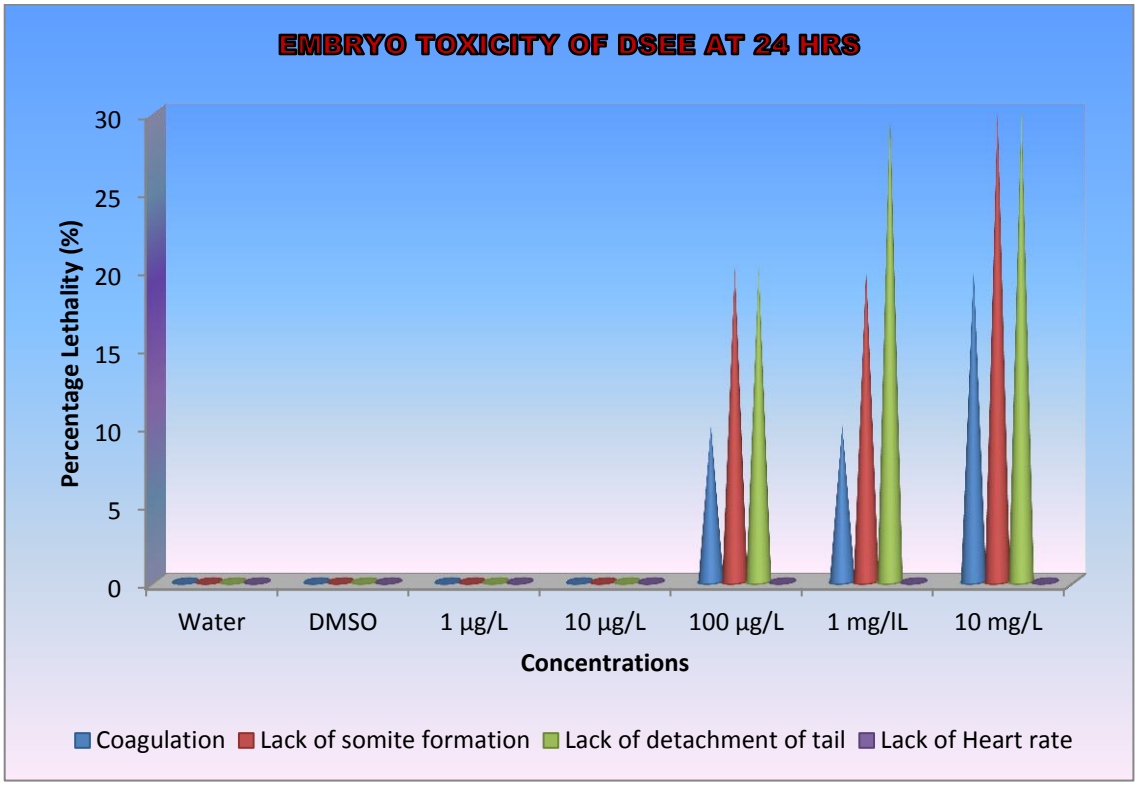
## FIG 9.5 EMBRYO TOXICITY OF DALPATEIN AT 24 HRS



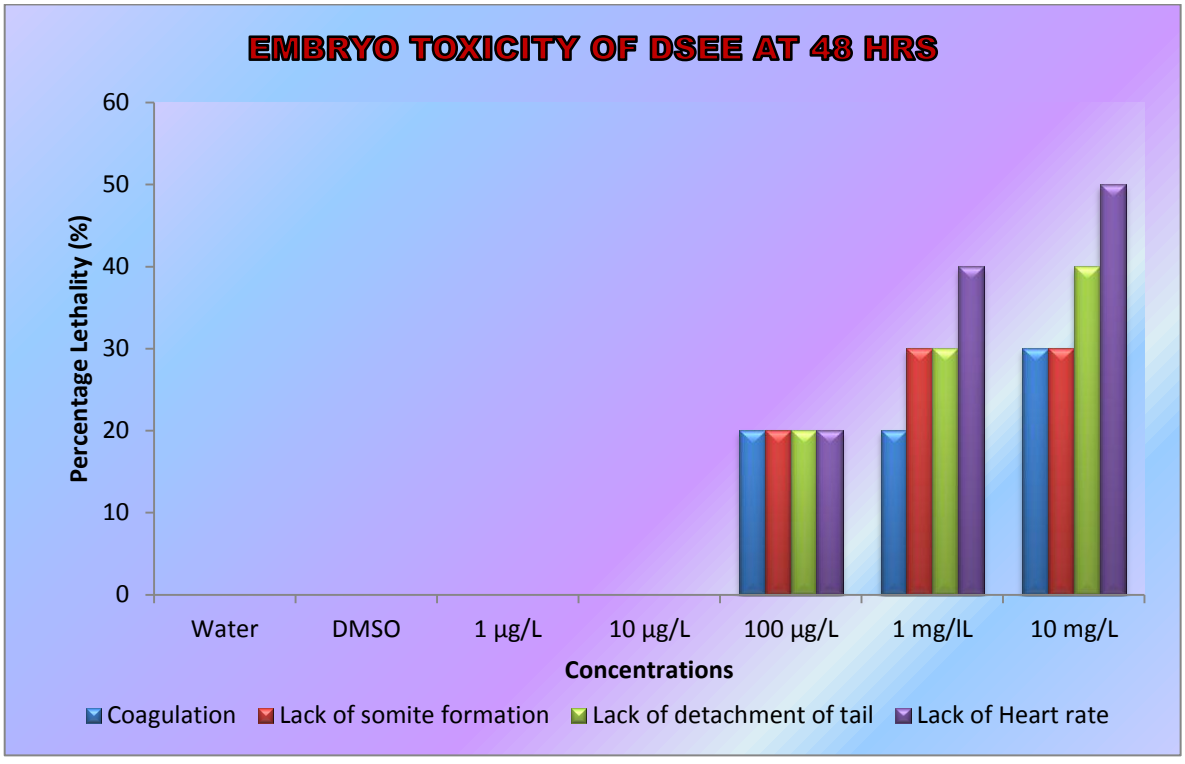

## FIG 9.6 EMBRYO TOXICITY OF DALPATEIN AT 48 HRS



## FIG 9.7 EMBRYO TOXICITY OF DSEE AT 24 HRS




## FIG9.8 EMBRYO TOXICITY OF DSEE AT 48 HRS



# FIG9.9 EMBRYO TOXICITY - PHOTOGRAPHIC REPRESENTATION

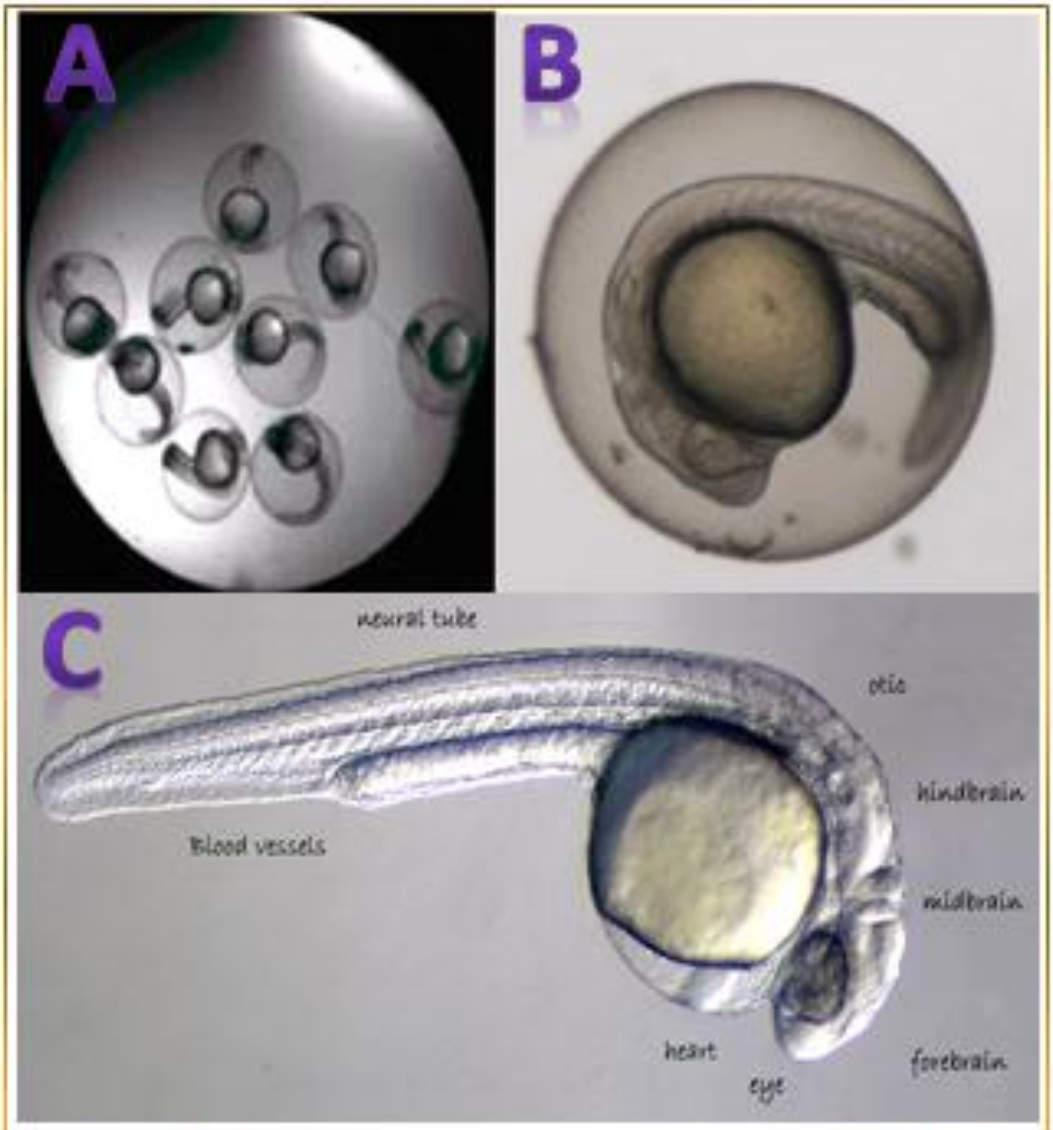
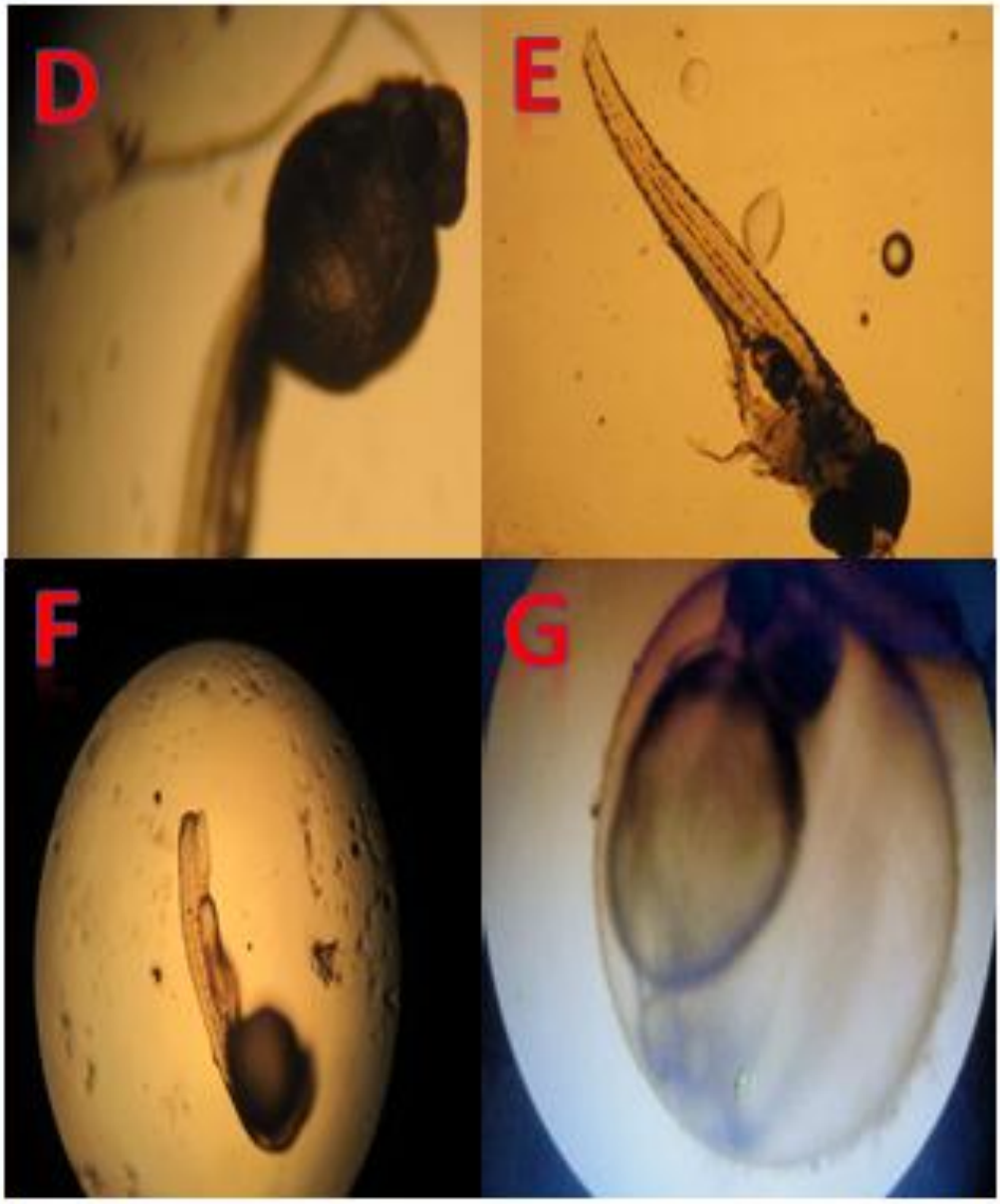
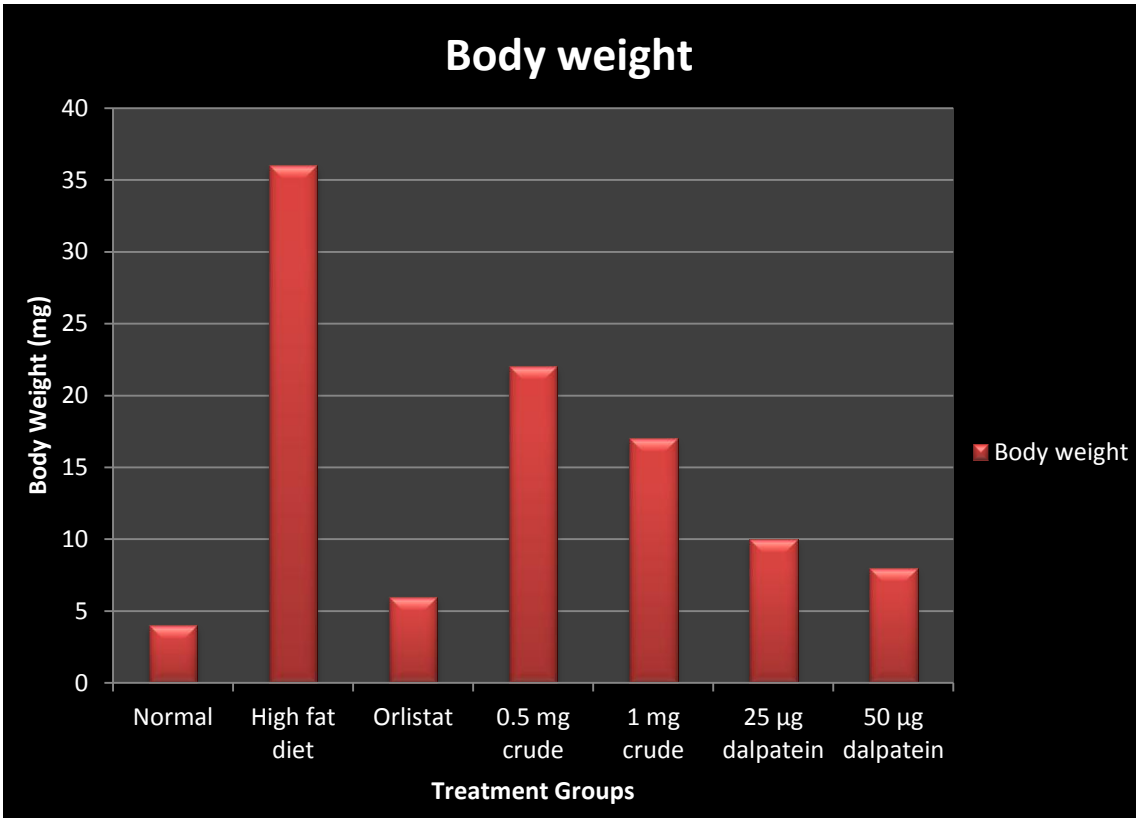



**FIG 9.9** (A) The Group of 24 hpf embryo at 96-well plate for the acute toxicity test. (B) A 48-hpf embryo before hatching. (C) A 72-hpf embryo (hatched larva).

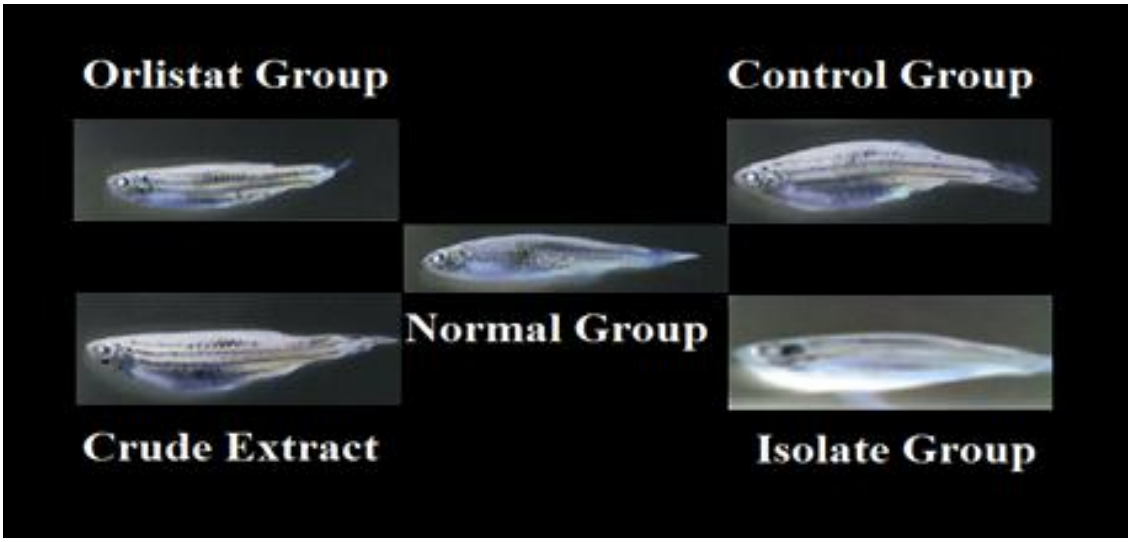



Toxicity studies of zebrafish embryos. (D) The 72-hpf larva with pericardial edema and bent tail hatched from embryo incubated with 1 mg of crude ethanolic extract. (E) The 72-hpf larva with tail detachment from embryo treated with 10 mg of dalpatein . (F) The 72-hpf larva with hook-like tail hatched from embryo incubated with 10 mg of crude ethanolic extract. (G) Normal embryo for heart rate measurement.

## FIG 10.1EFFECTS OF DSEE AND DALPATEIN ON BODY WEIGHTS IN ZEBRAFISH FED HIGH-FAT DIET




## FIG 10.2 EFFECT OF DRUG TREATMENT AND BODY WEIGHT- PHOTOGRAPHIC REPRESENTATION



# FIG 10.3 SERUM PROFILE OF ZEBRAFISH AFTER TREATMENT

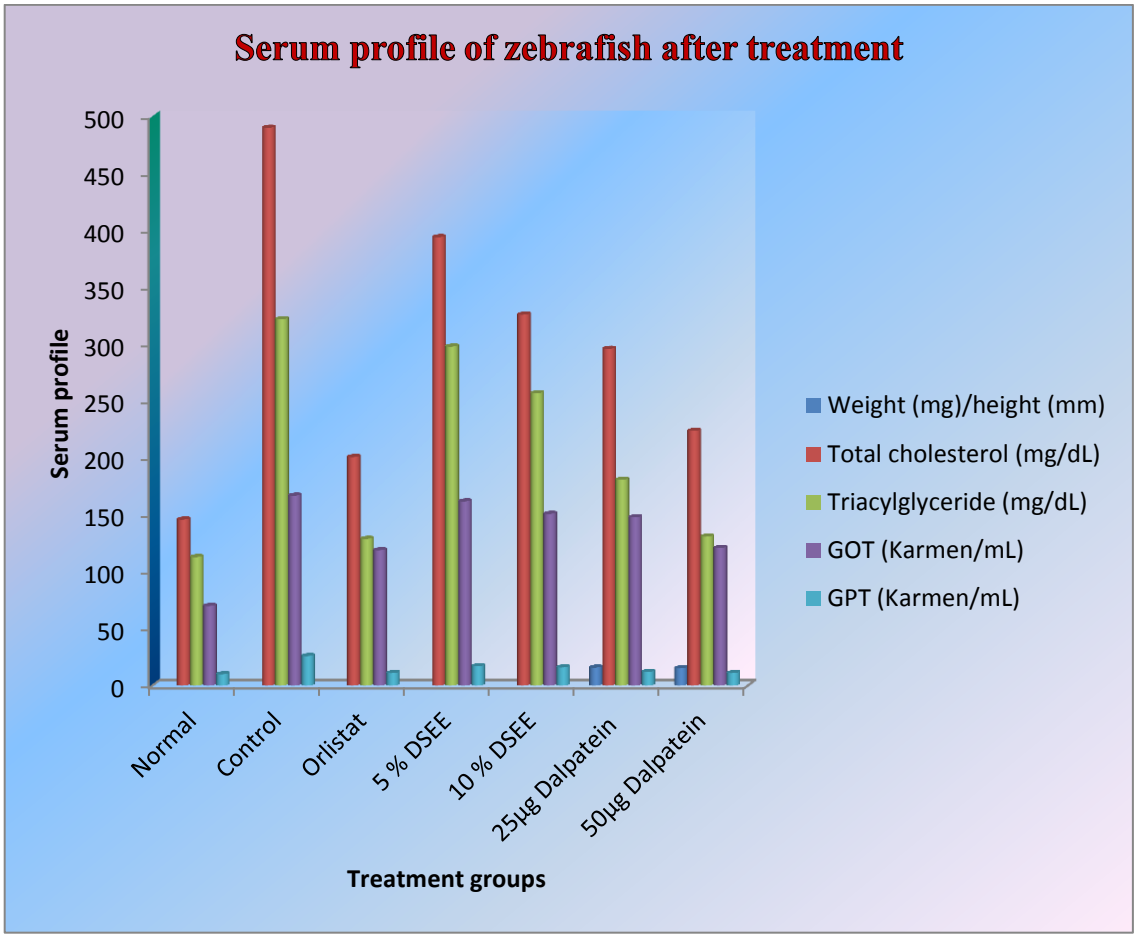
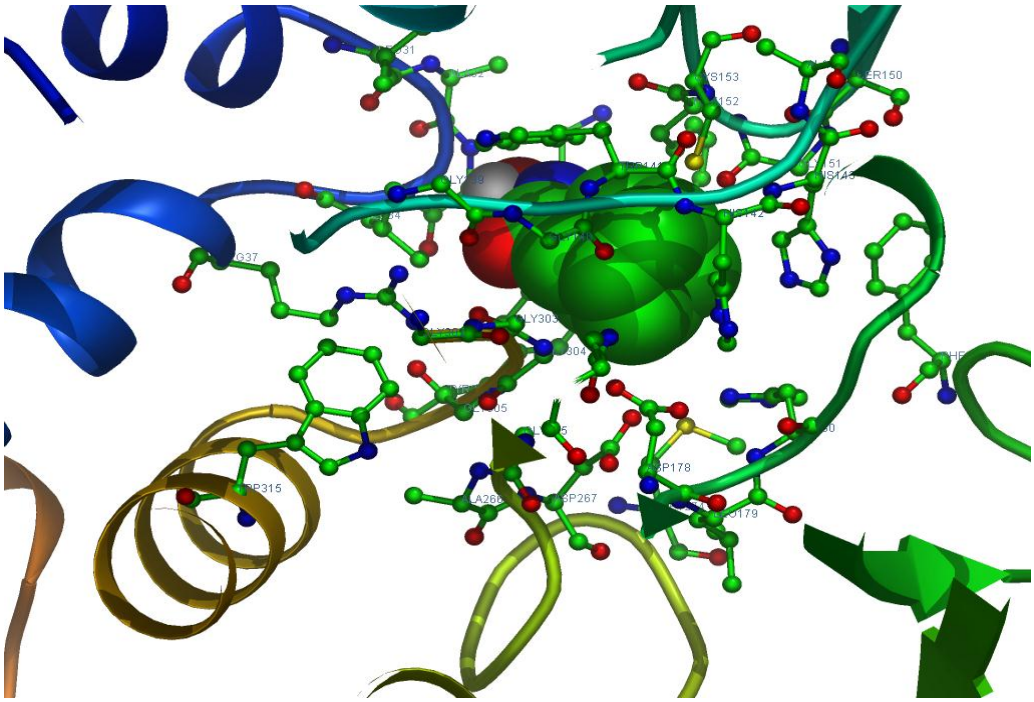
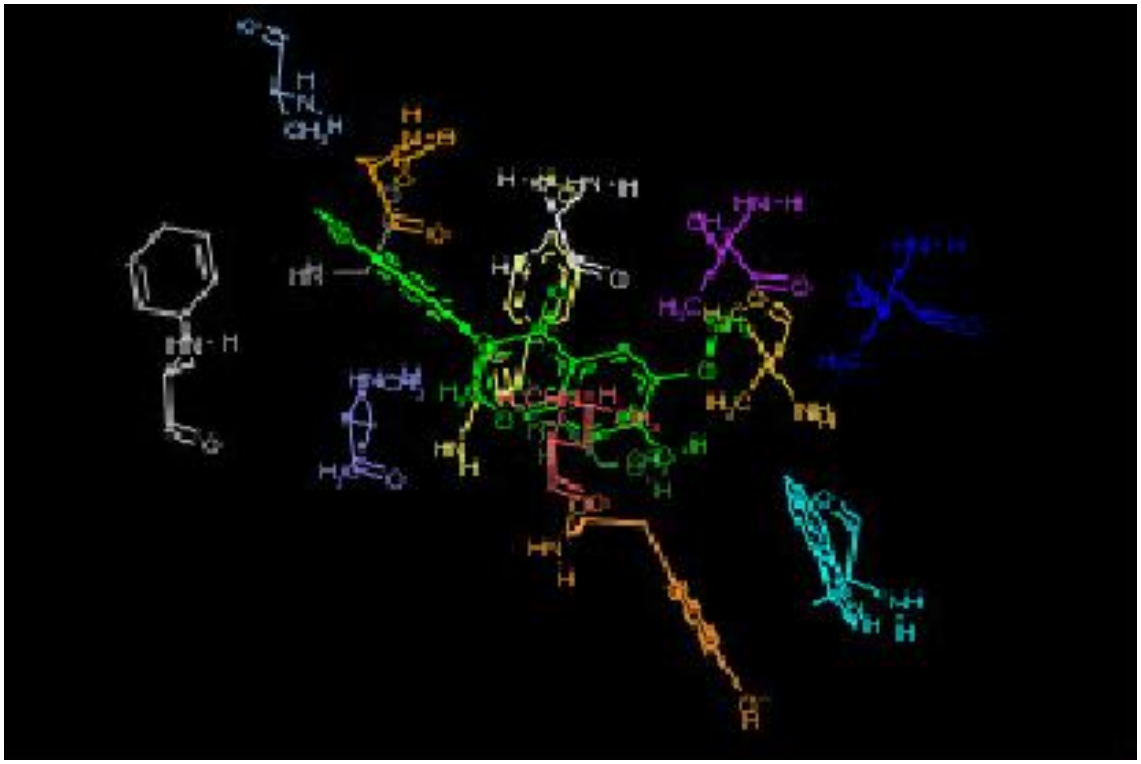
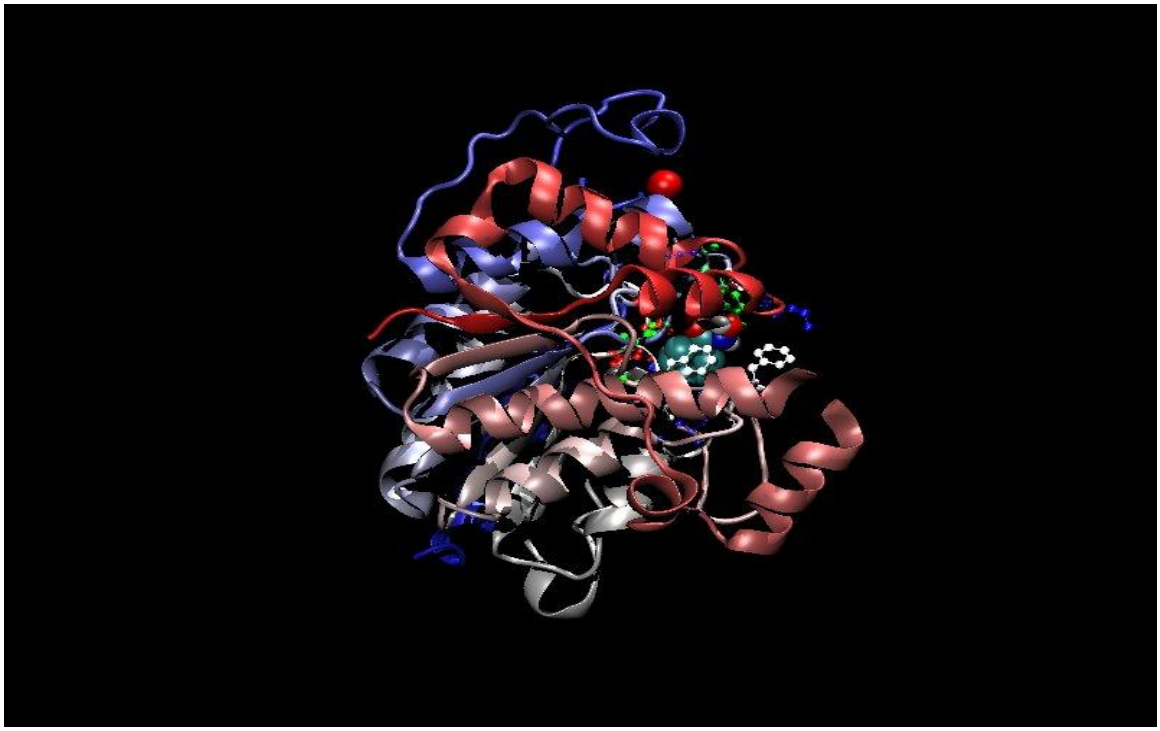
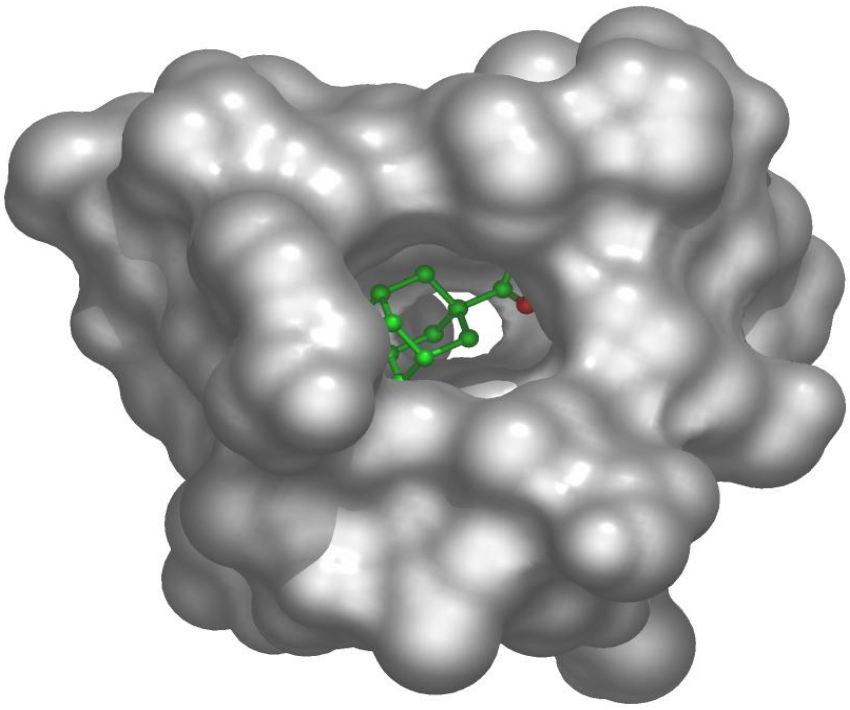


Fig.10.5. Molecular docking derived binding pose of the dalpatein in PL enzyme. The inhibitor is shown with four different representations used to derive the binding mode with one strong hydrogen bond with the hydrogen atom of the OH of Tyr306.









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